

cDNA CLONING AND ANALYSIS OF A flg VARIANT
FIBROBLAST GROWTH FACTOR RECEPTOR FROM
Xenopus laevis

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GANG CHEN



CDNA CLONING AND ANALYSIS OF
A *flg* VARIANT FIBROBLAST GROWTH
FACTOR RECEPTOR FROM *Xenopus laevis*

Gang Chen B.Sc.

A thesis submitted to the School of Graduate
Studies in partial fulfilment of the
requirements for the degree of
Master of Science

Faculty of Medicine.
Memorial University of Newfoundland.

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ISBN 0-315-91616-8

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Dedicated to my wife Chun Rong. Her love and sacrifice made this thesis work possible. To my wonderful daughter Elizabeth born during this thesis writing.

Abstract

1. A cDNA library was constructed with mRNA isolated from stage 8 *Xenopus* embryos.
2. A full length cDNA clone encoding a FGFR-1/*flg* gene was isolated by screening this cDNA library. It is designated XFGFR-A3.
3. The XFGFR-A3 clone was sequenced by dideoxynucleotide chain termination method on both strands with synthetic oligonucleotides. It is 3863 bp in length and is predicted to encode an 810 aa protein.
4. The XFGFR-A3 clone contains two dipeptide deletions, Val⁴²³-Thr⁴²⁴ and Pro⁴⁴¹Ser⁴⁴², which are different from the published XFGFR-1 sequence (Figure 8). The Pro⁴⁴¹Ser⁴⁴² deletion has been described previously, however, this is the first report of the Val⁴²³-Thr⁴²⁴ deletion in *Xenopus*. Both dipeptide deletions result in the removal of consensus phosphorylation sites for Protein Kinase C (PKC) which may have consequences on intracellular signal transduction and the regulation of embryonic development.
5. RT-PCR results showed that XFGFR-A3 was expressed at all stages of *Xenopus* embryonic development.
6. The RNase protection experiment showed that XFGFR-A3 is a minor form of the XFGFR-1 in all stages of *Xenopus* embryonic development. Like the wild type XFGFR-1, XFGFR-A3 is also uniformly expressed throughout *Xenopus* development.
7. The XFGFR-A3 genomic DNA sequence covering the VT deletion region was sequenced. The VT deletion is located at an exon/intron boundary and comparison with the cDNA sequence suggests that the XFGFR-A3 variant arises from the use of an alternate 5'splice donor site.
8. Expression vectors containing an insert covering the VT deletion region fused to the cDNA encoding GST were constructed by using XFGFR-A2 and XFGFR-A3. A PKC assay using the purified fusion proteins products showed that Thr⁴²⁴ of XFGFR-A2 can be phosphorylated by PKC *in vitro*.

Acknowledgements

I would like to extend my gratitude to my supervisor Dr. Laura Gillespie for giving me the opportunity to start my molecular biology research career in her lab. Her excellent guidance, encouragement and understanding helped me to overcome the difficulties encountered in my research work, course studies, and everyday life. I sincerely appreciated her generous support which was much more than she could give to me.

It is a pleasure to take this opportunity to express my sincere appreciation of all kinds of generous helps from Dr. Gary Paterno (include used car repairs). His professional helps kept me company all throughout my thesis works. Without him, the results presented here will never be the same.

I specially thanks Dr. Jon Church for his all kind of helps during my two year M.Sc. program in Terry Fox Cancer Researches Laboratories. Special thanks also go to Dr. Kenneth Kao for his helpful comments on writing the regional specification part of introduction. I thanks all the Terry Fox Labs members for their many helps, encouragement and friendships. I will never forget the good times shared with them. Special thanks to Mrs. L.T. Deng who assisted me sequenced the PCR amplified genomic DNA fragment of XPGFR-A3. Many thanks go to Ms. Ildiko Grandal and Mr. Nargendra Prasad for their helpful discussions and comments. I also wish to thanks Mrs. Ing Swie. Goping, Mr. Joe Teodoro of McGill University, and Mr. Xiaolong Yang for their expert computer assistance.

Fellowship from the School of Graduate Studies and department support from the Faculty of Medicine are greatly acknowledged.

I also wish to extend my thanks to Dr. Chet. Michalski and Ms. Pauline Cole for their cordial administration helps and supports all throughout my graduate program.

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List of Abbreviation and Symbols Used

aa	amino acid(s)
aFGF	acidic FGF
bFGF	basic FGF
bp	base pair(s)
cdna	complementary DNA
CSF-1	Colony Stimulating Factor 1 Receptor
DAG	Diacylglycerol
DEPC	diethylpyrocarbonate
DTT	Dithiothreitol
DMSO	Dimethyl sulphoxide
EDTA	ethylene diaminetetraacetic acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor
GST	glutathione S-transferase
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
Ig	Immunoglobulin
IP ₃	Inositol 1,4,5-triphosphate
IP ₃	isopropyl- β -D-thiogalactopyranoside
Kb	Kilobase
KD	Kilodalton
K-FGF	Kaposi's Sarcoma-FGF
MIFs	Mesoderm Inducing Factors
M-MLVRT	Moloney-Murine Leukemia Virus Reverse Transcriptase
mRNA	messenger RNA
NAM	Normal Amphibian Medium
nt	nucleotide(s)
ODC	Ornithine decarboxylase
PCR	Polymerase Chain Reaction
PDGFR	Platelet-derived Growth Factor Receptor
PIP ₂	Phosphatidylinositol bisphosphate
PKC	Protein Kinase C
PLC- γ 1	Phospholipase C γ 1
RTK	Receptor Tyrosine Kinase
RT-PCR	Reverse Transcription followed by PCR
SH2(3)	src homology domain 2(3)
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF- β	Transforming Growth Factor β
tRNA	transfer RNA
VT	Valine-Threonine dipeptide
XFGF	<i>Xenopus</i> Fibroblast Growth Factor

XFGFR	<i>Xenopus</i> Fibroblast Growth Factor Receptor
XTC	<i>Xenopus</i> tissue culture cell line

Chapter 1

Introduction

1.1. Regional Specification-the Core Problem in Embryonic Development Research

From egg to animal; from simplicity to complexity. Through more than a century of studies, embryologists now know that different tissues and organs come from different cells in the embryo. The different cells in the embryo were from corresponding regions in the egg where cell differentiation begins (Davison, 1990). It is a series of relatively simple developmental events that composed the whole embryonic development process. Each of these events is based on the former ones. The embryonic induction events, caused by cell-cell interaction among different embryonic regions, play very important roles in embryonic development. Regional specification, also called spatial organization or pattern formation, was primarily caused by embryonic induction events. As the core problem in current embryonic development studies, regional specification studies can tell us how cell

differentiation occurs and how body patterning forms during embryonic development.

1.2. The Advantage of *Xenopus laevis* System

Xenopus laevis is an ideal animal for developmental studies:

- 1). Its eggs are large (about 1.2 mm in diameter) and the embryos develop externally, so it is relatively easy to do microinjection and microdissection.
- 2). *Xenopus* eggs may be obtained in large numbers which makes it very easy to do most kinds of biochemical purification and analysis as well as morphological experiments.
- 3). By *in vitro* fertilization, large numbers of homogenously developing eggs can be obtained. In this way, the exact time of fertilization can be known. Also, the *Xenopus laevis* embryonic development stages have been well classified, thus making it easier to monitor the development process.
- 4). *Xenopus* embryos develop very quickly. But the embryo doesn't grow in size in the early stages. Since *Xenopus* embryos depend on their yolk for development, it is possible to add interesting substances into the salt culture medium (normal amphibian medium; NAM) to test their potential function in *Xenopus* embryonic development.

1.3. Early Events of *Xenopus Laevis* Embryonic Development

1.3.1. Facts About the *Xenopus laevis* Eggs

The unfertilized *Xenopus* eggs are radially symmetrical about the animal-vegetal axis (Gerhart, 1980). For example, there is a deep pigmentation on the animal hemisphere and the animal hemisphere contains small yolk granules compared to the vegetal hemisphere. The vegetal half has little pigment but many large yolk platelets. There are a few mRNAs and proteins specifically located in the animal or the vegetal hemisphere during oogenesis. There is evidence that some of the localized identified mRNAs and proteins have important roles in the early decisions of embryonic development (Lehmann and Nusslein-Volhard, 1986; Weeks et al., 1987, Steward, 1989; Ephrussi, et al., 1991; Wang and Lehmann, 1991; Cheung et al., 1992). The animal-vegetal axis is formed during oogenesis, but its origin is not known. Some experimental results have shown that the animal-vegetal-axis formation appears to involve selective transportation of proteins and RNAs to the future vegetal pole (Heasman and Wylie 1984; Gerhart and Keller 1986; Danilchik and Gerhart 1987).

1.3.2. Events after Fertilization

1.3.2.1 Regional Specialization in the First Cell Cycle

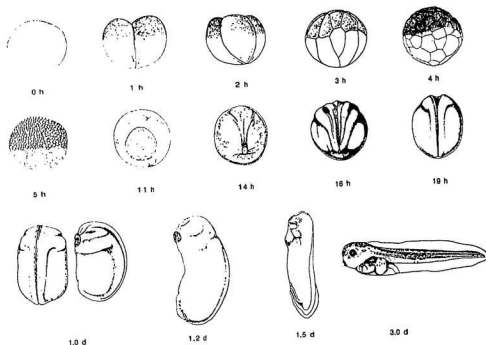
Before first cleavage, about an hour after fertilization, the rigid embryo surface cortex rotates by about 30° relative to the inner cytoplasm (Figure 1, Gerhart *et al.*, 1989). This is a very important step in the establishment of dorsal-ventral polarity. This cortical/cytoplasmic rotation produces the grey crescent on the dorsal side opposing the sperm entry site (Vincent *et al.*, 1986; Gerhart *et al.*, 1989). In many species, the grey crescent results from the visibility of pigmented animal cytoplasm through the rotated vegetal cortex. The cortical rotation generates differences in the dorsal and ventral cells which result in their different abilities to induce a dorsal axis (Gimlich and Gerhart, 1984; Gimlich, 1986; Kageura, 1990). Cortex rotation, which provides the dorsoventral specification, is brought about by cytoplasmic movements inherent to the structure of the egg but not by the effect of sperm entrance or gravity (Gerhart, *et al.*, 1989). The importance of the cortical/cytoplasmic rotation was demonstrated by ultraviolet (UV) irradiation of the vegetal half of embryo. When the

vegetal half of the embryo is irradiated with ultraviolet light before cortex rotation, the rotation is inhibited (Manes and Ellison 1980; Vincent and Gerhart 1987). The resulting embryos can cleave and gastrulate but develop no dorsal or anterior structures (Scharf and Gerhart 1980, 1983).

1.3.2.2. Early *Xenopus* Development Process

In the frog embryo, the first cleavage (Figure 1) begins at the animal pole and end at the vegetal pole and separates the embryo into right and left sides. The second cleavage also begins at the animal pole and at right angles to the first cleavage plane. At the four cell stage, the individual cells have been programmed for a different fate in the future body pattern. The third cleavage is along the equator, separating animal from vegetal hemisphere. The third cleavage is slightly toward the animal pole resulting in smaller sized cells in the animal hemisphere than those in the vegetal hemisphere. This size difference between animal and vegetal hemisphere cells is perpetuated throughout subsequent cleavages. As cleavage proceeds, a large central cavity called a blastocoel forms surrounded by a layer of cells. The embryo at this stage is called a blastula. The blastula stage

Figure 1. The *Xenopus laevis* embryonic development chart measured in hours (h) or days (d) after fertilization. Blastula stage occurs at 4 to 5 h after fertilization. (Modified from Nieuwkoop and Faber staging table. 1975)



embryo undergoes extensive rearrangement called gastrulation, a process which transforms the vertebrate embryo and produces a distinctive body plan with three tissue layers: ectoderm (outer layer); mesoderm (intermediate layer); endoderm (inner layer). Gastrulation in *Xenopus* changes the blastula into a three layered structure with anteroposterior and dorsoventral axes. These three germ layers will give rise to different tissues and organ systems in the adult animal. Ectoderm gives rise the nervous system and epidermis. Mesoderm develops into cardiac and skeletal muscle, notochord, bone and cartilage, connective tissue, kidney blood and mesenchyme. Endoderm produces the digestive tract and associated organs, such as liver and pancreas. It is a series of induction events that control the embryonic development. One very famous experiment performed by Spemann and Mangold (1924) showed that transplantation of a small region of a gastrula stage amphibian embryo into a new location in a host embryo could induce the formation of a second body axis in the host embryo. This small region of embryo, located above the dorsal blastopore lip, is called " Spemann's orgnizer ". It can organize the rest of the embryo to develop the full dorso-anterior axial structures. Three induction events were believed to have important roles in embryonic development. The first induction event occurs in

the blastula stage and is called mesoderm induction. The second induction is called dorsalization and affects the dorsal-ventral specification of the mesoderm (Dale and Slack, 1987). The third induction, also known as neural induction, occurs during gastrulation and produces the nervous system (Sharpe, 1990).

1.4. Mesoderm Induction

1.4.1. Mesoderm Induction and Models

The early blastula consists of animal and vegetal cells. When in contact, vegetal cells induce animal cells to form the mesoderm. This induction event is one of the earliest cell-cell interactions to occur in amphibian development (Green and Smith, 1990). The cells that will form the mesodermal germ layer are located around the equator of the embryo. It is generally believed that an inductive signal is emitted from the neighbouring vegetal hemisphere cells and acts on neighbouring equatorial cells to induce them to become mesodermal cells. The *Xenopus* mesoderm induction was first described by Sudarwati and Nieuwkoop (1971). Their findings demonstrated that mesoderm formation relies on an interaction between animal and vegetal cells. When the blastula stage embryo is dissected and cultured, the animal

pole cells form epidermis only, while the vegetal pole cells form poorly differentiated endoderm. However, cultured together, animal and vegetal pole cells can form a variety of mesodermal tissues. Mesoderm has a dorsal-ventral polarity. Work done by Boterenborond and Nieuwkoop (1973) showed that vegetal pole cells from the dorsal side of the blastula tended to induce dorsal cell types such as notochord and muscle while lateral and ventral vegetal blastomeres induced blood, a characteristic ventral cell type, along with mesenchyme and mesothelium. Ventral vegetal blastomeres induce little or no muscle from animal pole cells. This demonstrates that at least two different types of induction can occur: ventral mesoderm induction and dorsal mesoderm induction.

Slack and colleagues proposed a " three signal model" for mesoderm formation (Smith and Slack, 1983; Slack et al., 1984). In this model, the first signal is released uniformly from the ventral and lateral vegetal hemisphere, creating a ring of ventral-type mesoderm in most of the equatorial zone. A second signal originating in the dorsal vegetal region induces the formation of the most dorsal type of mesoderm including Spemann's organizer, in the overlying dorsal equatorial zone. The Spemann's organizer sends a third

signal across the mesoderm to convert the initial ventral mesodermal cells to a variety of different intermediate mesodermal cell types. Today, more and more evidence suggests that the formation and patterning of mesoderm is the result of a number of overlapping signals rather than three separated events. To expand the three signal model, a synergistic model for mesoderm induction was proposed (Kimelman *et al.*, 1992). This model emphasizes that the inducing signals work synergistically. For example, FGF by itself can only induce ventral mesoderm. By synergizing with the Wnt-like factors, FGF can also induce dorsal mesoderm. So in the synergistic model, the first step for dorsal ventral patterning is activation of a wnt-like factor by cortical rotation in a broad dorsal region of the egg, with maximum activity at the dorsal midline of the equatorial region. This activity can determine the competence of the animal hemisphere to respond to mesoderm inducing signals such as FGF and activin B.

1.4.2. Mesoderm Inducing Factors

The most extensive and comprehensive studies concerning about the mesoderm induction events in amphibia were completed within the past ten years. Slack *et al.* (1987)

demonstrated that FGF can mimic the vegetal inducing signal and induce explanted tissue from *Xenopus* embryos to form mesoderm. Another family of polypeptide growth factors that have been implicated in mesoderm induction is the TGF- β (transforming growth factor- β) superfamily which includes the activins (Smith, 1987; Smith *et al.*, 1990; Thomsen *et al.*, 1990) and bone morphogenetic protein 4 (BMP-4) (Koster *et al.*, 1991; Dale *et al.*, 1992; Jones *et al.*, 1992).

The mesoderm-inducing capacity of these factors has been tested primarily by addition of potential mesoderm-inducing factors to blastula stage explants of the animal hemisphere (the " animal cap ") which normally develop into ectodermal tissues (Smith 1987.). These *in vitro* experiments have demonstrated that FGFs tend to induce ventral mesoderm while activin primarily induces dorsal mesoderm (Slack *et al.*, 1987; Paterno *et al.*, 1989; Smith, 1987; Smith *et al.*, 1990).

A second class of molecules, including *Wnt* (Christian *et al.*, 1991; Smith and Harland, 1991; Christian *et al.*, 1992 *Noggin* (Smith and Harland, 1992) and lithium (Kao *et al.*, 1986; Slack *et al.*, 1988; Kao and Elinson, 1989) differs from the general mesoderm inducer families mentioned above. They can not directly induce mesoderm on their own, instead,

they modify the target cell's response to the mesoderm inducing factors. For example, FGF alone is not able to induce notochord in ectodermal explants, but can do so in combination with *Xwnt-8* (Christian et al., 1992; Kimelman et al., 1992). *Wnt* family members can also cooperate with activin (Sokol and Melton, 1992). Mesoderm induction is regulated temporally and spatially: both the appearance of inducer molecules and the acquisition of competence, which is the ability of a tissue to respond to the inductive signal, are timed precisely in the appropriate region of the embryo.

It will be a crucial but difficult task to show which of these factors are really involved in mesoderm induction events during embryonic development. Criteria must be set for the identification of the natural mesoderm inducing factors and should include: 1. The potential mesoderm-inducing factor must be expressed at a high enough concentration at the right time, right stage. 2. The purified protein must show the expected mesoderm-inducing function; 3. Blocking of the factor should cause inhibition of the mesoderm induction *in vivo* . Of these three aspects, the last one is the most difficult one to demonstrate. Mesoderm-inducing factors begin to act as early as the 64 cell stage. Because zygotic transcription has not begun start at this time, mesoderm-

inducing factor mRNA or protein must be present in the egg (Shuttleworth and Colman, 1988; Slack et al., 1992). Evidence to date suggests that many of the MIFs and modifying factors mentioned above have some role to play in mesoderm induction. The work presented in this thesis is focussed on FGF and mesoderm induction in *Xenopus* and, therefore, the remainder of this introduction will be concerned with the FGFs and their receptors.

1.5. FGFs and Mesoderm Induction

1.5.1. The FGF Family Members

The fibroblast growth factors (FGFs) are multi-functional polypeptides that are expressed in a variety of embryonic and adult cell types and are involved in many important developmental processes including embryonic induction of mesoderm, angiogenesis, chemotaxis, proliferation, and neuronal maintenance (Burgess and Maciag, 1989; Rifkin and Moscatelli, 1989; Gospodarowicz, 1990).

Fibroblast growth factor was first identified from bovine brain extracts by its ability to stimulate proliferation of BALB/c 3T3 fibroblasts (Gospodarowicz, 1974). Now the FGF multigene family is known to consist of nine related members

which are evolutionarily highly conserved (Armelin, 1973; Gospodarowicz *et al.*, 1974; Michael *et al.*, 1992). These FGF family members have 155-268 amino acids (110-150 KDa) and have 33-65 % homology at the amino acid level. As shown in Table 1, The FGF multigene family members cloned so far includes aFGF (FGF-1) (Jaye, *et al.*, 1986), bFGF (FGF-2) (Abraham *et al.*, 1986 b.), int-2 (FGF-3) (Moore *et al.*, 1986; Dickson and Peters, 1987), kFGF (FGF-4, also called ks/hst FGF) (Delli Bovi *et al.*, 1987), FGF-5 (Zhan *et al.*, 1988), FGF-6 (Marics *et al.*, 1989 2), KGF (FGF-7) (Finch *et al.*, 1989), FGF-8 (Tanaka *et al.*, 1992) and FGF-9 (Miyamoto *et al.*, 1993). Another FGF called XeFGF, with a unique expression pattern has been cloned from *Xenopus* embryos recently (Isaacs *et al.*, 1992).

The highest degree of similarity is between acidic FGF (aFGF) and basic FGF (bFGF), the two oldest members of FGF family, both can stimulate proliferation of cells of mesenchymal, epithelial, and neuroectodermal origin. The cDNA of acidic and basic FGFs encode 155 amino acid proteins with 55 % sequence homology. The 155-amino acid protein is encoded by three exons (Abraham *et al.*, 1986 a, b). aFGF and bFGF and FGF-9 are the only three FGF family members which lack a hydrophobic secretion signal sequence located on the N-

Table 1 . FGF family members

aFGF (FGF-1)	(Jaye, et al., 1986)
bFGF (FGF-2)	(Abraham et al., 1986 b.)
INT-2 (FGF-3)	(Moore et al., 1986)
	(Dickson and Peters, 1987)
kFGF (FGF-4, ks/hst FGF)	(Delli Bovi et al., 1987)
FGF-5	(Zhan et al., 1988)
FGF-6	(Marics et al., 1989)
KGF (FGF-7)	(Finch et al., 1989)
FGF-8	(Tanaka et al., 1992)
FGF-9	(Miyamoto et al., 1993)
XeFGF	(Isaacs et al., 1992)

terminal of protein. *int-2* and hst/kaposi FGF (kFGF) are proto-oncogenes products. *int-2* FGF is expressed in the brain of the adult mouse and its expression is developmentally regulated during embryogenesis. kFGF is a proto-oncogene product isolated from human stomach cancers and in Kaposi's sarcoma. It is a 206 amino acid protein with 40 % homology to bFGF. The *int-2* and KFGF genes are located in close proximity in the human and mouse genomes. They are co-expressed in several human cancers. FGF-5 is a 268 amino acid protein which was also first identified as an oncogene product. FGF-6 is a 198 or 208 amino acid protein, depending on the initiation site used. Keratinocyte growth factor (KGF) is a mitogen specific for epithelial cells. KGF receptor can bind KGF and aFGF with high affinity but binds bFGF with low affinity. XeFGF is a new member of the fibroblast growth factor family isolated from a *Xenopus laevis* embryo cDNA library. It is closely related to both mammalian kFGF (FGF-4) and FGF-6. Two sequences of XeFGF were obtained that differ by 11% at the amino acid level. This raises the possibility that they represent pseudotetraploid variants. (Isaacs et al., 1992). FGF-8, also called androgen-induced growth factor (AIGF) was purified from a conditioned medium of an androgen-dependent mouse mammary carcinoma cell line (SC-3). An FGF-8 cDNA encodes a 215 aa protein with a putative

signal peptide, and shares 30-40% homology with the known members of the FGF family. It has been shown that the androgen-induced growth of SC-3 cells is mediated in an autocrine manner by FGF-8, secreted by the tumor cells themselves in response to hormonal stimuli (Tanaka *et al.*, 1992). Glia-activating factor (GAF) or FGF-9 is a novel heparin-binding growth factor purified from the culture supernatant of a human glioma cell line. Human FGF-9 cDNA clone encoded a polypeptide consisting of 208 amino acids. It shares 30 % similarity with other members of the FGF family. FGF-9 was found to have no typical signal sequence in its N terminus like those in aFGF and bFGF. Both aFGF and bFGF are known not to be secreted from cells in a conventional manner. However, FGF-9 was found to be secreted from cells after synthesis despite its lack of a typical signal sequence. FGF-9 cDNA from rat has also been cloned. Its sequence is highly conserved between rat and human. Expression of the FGF-9 gene could only be detected in the brain and kidney of the adult rat suggesting that FGF-9 plays a different physiological role from other members of FGF family (Miyamoto *et al.*, 1993).

There is 84 % homology between *Xenopus* and human bFGF (Kimelman *et al.*, 1988) and 79% between *Xenopus* and human

FGFR 1 (Friesel. R. and Dawid, I.B. 1991). Both mouse and human FGF can interact with *Xenopus* FGF receptors to induce mesoderm in *Xenopus* explants. Therefore, experimental results obtained from studying the *Xenopus* system will provide useful information for higher vertebrates.

1.5.2. The Presence of FGF mRNA and Protein in *Xenopus* Embryos

Both FGF protein (Slack and Isaacs, 1989) and mRNA (Kimelman et al., 1988) are present in the *Xenopus* embryo. The first FGF cDNA clone was isolated by Kimelman and Kirschner (1987) from a *Xenopus* oocyte library. This cDNA clone contained sequences closely related to human and bovine bFGF. It was later shown that this cDNA clone contained just a short open reading frame encoding a peptide domain homologous to the third exon of mammalian bFGF; sequences homologous to the first and second exons were missing. A 4.3 kb cDNA clone was isolated later and it encoded a protein of 155 amino acids and share 89 % identity with human bFGF at the amino acid level (Kimelman and Kirschner, 1988). When protein from this cDNA was synthesized in a T7 expression system and purified by heparin-Sepharose chromatography it

was shown to be as effective as bovine bFGF in inducing muscle differentiation from isolated *Xenopus* animal caps.

Two research groups have demonstrated that bFGF protein is present in the *Xenopus* embryo (Kimelman et al., 1988; Slack and Isaacs, 1989). By passing extracts from eggs and blastulae through heparin-Sepharose columns and eluting the bound material with high concentrations of NaCl, Slack and Isaacs (1989) showed that the eluted material had mesoderm-inducing activity. This activity could be blocked by antibodies to bFGF, but not to aFGF or TGF- β . The active fractions purified from HPLC heparin affinity column can be recognized by an bFGF antibody on Western Blots. It is estimated that the total amount of FGF present in the *Xenopus* embryo is between 7ng-70ng/ml. Immunostaining by using antibodies to both acidic and basic FGF showed that both aFGF and bFGF are present in oocytes and early embryos. Immunostaining was predominantly intracellular and was concentrated in the marginal zone and vegetal pole throughout cleavage and blastula stages (Shiurba et al., 1991). Taken together the evidence suggest that both FGF mRNA and protein are present in *Xenopus* embryos and actively involved in mesoderm induction *in vivo*.

Varying sizes of bFGF transcripts have been found in different species (Abraham *et al.*, 1986 a, b; Kimelman *et al.*, 1988). Different molecular weight forms of bFGF corresponding to approximately 18, 22.5, 23, and 24 KDa bFGF protein can be translated from a single human bFGF mRNA transcript (Florkiewicz and Sommer, 1989; Prasts *et al.*, 1989). A 1.5 Kb bFGF antisense transcript, which may have an important role in the regulation of bFGF expression, was found during *Xenopus laevis* oogenesis and embryogenesis (Kimelman and Kirschner, 1989; Volk *et al.*, 1989).

1.6. Fibroblast Growth Factors Receptors (FGFRs) and Intracellular Signal Transduction

1.6.1. Growth Factors and Tyrosine Kinase Activity

Growth factors are crucial regulatory molecules for multicellular organisms. Many growth factors are pleiotropic. Most act by binding to and activating cell surface receptors with an intrinsic protein tyrosine kinase activity. Most receptor tyrosine kinase possess a large glycosylated,

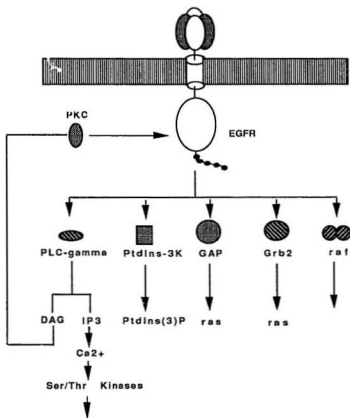
extracellular ligand binding domain, a single hydrophobic transmembrane region, and a cytoplasmic domain that contains a juxtamembrane domain and a tyrosine kinase catalytic domain. The tyrosine kinase domain is the most highly conserved portion of all receptor tyrosine kinase molecules. It contains a consensus sequence Gly X Gly XX Gly X (15-20) Lys (Yarden and Ullrich, 1988; Schlessinger, 1988; Hanks et al., 1988) for binding ATP. The transmembrane domain of receptor tyrosine kinases is separated from the cytoplasmic catalytic domain by juxtamembrane sequences that vary from receptor family to family, but are conserved between members of the same receptor family subclass. It has been suggested that this juxtamembrane region is involved in the regulation of receptor function (Ullrich and Schlessinger, 1990).

Some recent experimental evidence indicates that the protein tyrosine kinases (PTKs) have a important role in developmental processes including migration, proliferation and differentiation. Many of these PTKs have been shown to be expressed at high levels during embryonic development in a localized region and at much higher levels than are expressed in adults (Pawson and Bernstein, 1990). Normal function of certain PTKs has been shown to be crucial to normal development (Pawson and Bernstein, 1990) as disruption of

PTK function during embryogenesis can result in major phenotypic changes.

Ligand binding to the extracellular domain of a growth factor receptor induces receptor dimerization, resulting in receptor autophosphorylation by an intermolecular mechanism (Ullrich and Schlessinger, 1990). Intracellular substrates, which possess catalytic activity regulated by tyrosine phosphorylation, then interact with and are phosphorylated by the activated growth factor receptors (Figure 2). Intracellular substrates that have been identified to date include phospholipase C- γ (PLC- γ), p21^{ras}, GTPase-activating protein (GAP), Grb2 (Egan *et al.*, 1993; Li, N. *et al.*, 1993), and the putative regulatory subunit of phosphatidylinositol (PI) 3'-kinase (p85) (Cantley *et al.*, 1991). These substrates contain SH2 or SH3 (src homology regions 2 or 3) domains, noncatalytic domains of 100 aa. conserved in a series of cellular signal transduction proteins (Cantley *et al.*, 1991; Koch *et al.*, 1991). For example, Grb2 in mammals functions by recruiting a Ras activator, mSos, to the receptor to form a stable complex. Recruitment of Sos1 facilitates Ras activation and subsequent signal transmission down the Ras-dependent kinase cascade. Grb2 binds to activated receptors by the SH2 domain, and to

Figure 2. Diagrammatic representation of receptor tyrosine kinases and intracellular signal transduction pathways.



Sos by the SH3 domain. Receptor-associated Sos provokes GDP-GTP exchange on Ras triggering a cascade of serine-threonine kinases that send trophic signals to the nucleus.

1.6.2. FGF Receptor Signal Transduction Pathway

1.6.2.1. High Affinity and Low Affinity FGF

Receptors

FGFs rely on FGF receptors (FGFRs) to transmit the signal across the plasma membrane. There are two types of FGFRs. Low affinity binding sites, represented by heparan sulfate in the extracellular matrix or on the cell surface and high affinity binding sites, represented by transmembrane receptors (Burgess and Maciag 1989). FGF binding to heparan sulfate is a pre-requisite for interaction of FGF with high affinity transmembrane receptors. In the extracellular matrix, soluble heparan sulfate molecules can serve as a FGF carrier to protect it from being proteolysed (Folkman, et al., 1988).

1.6.2.2. Structure of High Affinity FGF Receptors

FGF receptor tyrosine kinases are similar to other growth factor receptors such as the platelet-derived growth factor (PDGFR) receptor and the epidermal growth factor receptor (EGFR). The unique features of the FGF receptor tyrosine kinase are as follows: an extracellular region with three Ig (immunoglobulin)-like domains, a relatively long juxtamembrane region, a kinase catalytic domain split by a 14 amino acid sequence and a short carboxyl terminal tail (Michael et al., 1992).

All high affinity FGF receptors encode a transmembrane protein which consists of 800-822 amino acids with 6-9 N-linked glycosylation sites in the extracellular domain. The N-terminus contains a 18-24 amino acid signal sequence, followed by a 346-356 amino acid extracellular domain containing three Ig-like (Ig) domains with a very acidic region between first and second Ig-like domain. The 21 amino acid transmembrane domain is followed by a 410-425 amino acid cytoplasmic domain consisting of 74-81 amino acid juxtamembrane region, the intracellular tyrosine kinase domain with 14 amino acid insertion, and a carboxyl-terminal

of 59-69 amino acid residues (Figure 3).

1.6.2.3. FGF Receptor Dimerization and Receptor Signal Transduction

The EGF receptor ligand binding induces the dimerization of EGF receptors. The EGF receptor dimerization causes EGF receptor conformational change and leads to receptor autophosphorylation in the cytoplasmic region by an intermolecular mechanism (Ullrich and Schlessinger, 1990). This results in EGF receptors higher ligand binding affinity and elevated protein tyrosine kinase activity. It is speculated that the same mechanism is used by FGFR. FGF receptor tyrosine kinases catalyze the phosphorylation of tyrosine residues on their own intracellular domains as well as on substrates (Honegger et al., 1989; Ballotti et al., 1989). Of the known intracellular PTK substrates only phospholipase C- γ (PLC- γ) has been shown to interact with the FGFR. Substrate PLC- γ can catalyze the breakdown of the membrane phospholipid, phosphatidylinositol biphosphate to inositol 1,4,5-triphosphate (IP₃) and diacylglycerol (DAG). These second messengers, as well as other serine/threonine

Figure 3. Diagrammatic representation of the FGFR structure. SP= signal peptide, I= first Ig-like domain, AR= acidic residue cluster, II= second Ig-like domain, III= third Ig-like domain, TM= transmembrane region, JM= juxtamembrane region, TKI= tyrosine kinase domain I, KI= kinase insert sequence, TKII= tyrosine kinase domain II.



protein kinase can release calcium from intracellular stores and activated protein kinase C (PKC).

1.6.3. FGF Receptors in *Xenopus* Embryonic Development

1.6.3.1. FGF Receptor Family Members

The FGF receptor was originally isolated from chicken embryos (Lee *et al.*, 1989) and shown to have homology to a partial human cDNA clone which had been named *flg* (*fms*-like gene) (Ruta *et al.*, 1988). Since then FGFRs have been cloned and characterized from chicken, human, mouse and *Xenopus* (Michael *et al.*, 1992). To date four different FGFR genes, *flg* (*fms* like gene) or FGFR1 (Ruta *et al.*, 1988), *bek* (bacterial expressed kinase) or FGFR2 (Dionne *et al.*, 1990), FGFR3 (Keegan *et al.*, 1991), FGFR4 as well as a number of receptor variants have been cloned (Table 2; Michael *et al.*, 1992). These FGF receptors encode proteins that have 800-822 amino acid residues and share 56-92 % identity at the amino acid level.

Table 2. FGFR family members

FGFR1/ <i>flg</i> (fms-like gene)	Ruta <i>et al.</i> , 1988
FGFR2/ <i>bek</i> (bacterial expressed kinase)	Dionne <i>et al.</i> , 1990
FGFR3	Keegan <i>et al.</i> , 1991
FGFR4	Machael <i>et al.</i> , 1992

1.6.3.2. FGF Receptors and Mesoderm Induction

Current evidence indicates that FGF and the FGFR signalling system are involved in mesoderm induction events in *Xenopus* embryonic development (Amaya et al., 1991; Jessell et al., 1992; Kimelman et al., 1988; Slack et al., 1987; Whitman and Melton, 1992; Ryan and Gillespie 1993, submitted). FGFs, such as bFGF, int-2 and XeFGF, can mimic the mesoderm-inducing activity of vegetal pole cells in the early blastula and, hence, may be a component of the natural inducer (Slack et al., 1987; Kimelman et al., 1988). The FGFR1 is activated during mesoderm induction by vegetal cells providing direct evidence that FGF is a component of the vegetal inducing signal (Ryan and Gillespie 1993, submitted). The importance of FGFR signalling in embryonic development was recently demonstrated by Amaya et al., (1991). These investigators demonstrated that the injection into embryos of cRNA encoding a dominant-negative mutant form of FGFR1 that lacks the intracellular tyrosine kinase domain can cause significant reduction in mesodermal structures of tadpoles. Explanted animal pole ectoderm derived from embryos expressing the truncated receptor failed to form mesoderm in response to FGF-2. This effect could be reversed by forced overexpression of the wild-type receptor. These data clearly

demonstrate an important role for the FGFs and their receptors in embryonic mesoderm formation . However, the mechanisms by which this occurs remains to be elucidated (Amaya *et al.*, 1991).

1.6.3.3. The Expression Pattern of FGF Receptors

Distinct patterns of expression of each FGF receptor were found in different species, and tissues, suggesting a functional specificity. Recent studies show that FGFR1/*flg* mRNA were highly expressed in the migrating embryonic mesoderm of gastrulation mouse embryo (Yamaguchi *et al.*, 1992).

Affinity labelling was used to detect the presence of FGFR protein on the surface of different types of cells. Two major bands, 125 Kd and 145 Kd, were identified in most cell types. The 145 Kda protein had a higher affinity for bFGF, while the 125 Kda protein had a apparently higher affinity for aFGF (Neufeld *et al.*, 1986). Two FGF receptors (130 kd, 140 kd respectively) were identified in *Xenopus* blastula by using the same approach (Gillespie *et al.*, 1989). Despite the fact that cDNA clones of four distinct FGFR genes have been

characterized, it remains unclear whether these two FGFR proteins on cell surface are the products of different genes, alternatively spliced products of the same gene or result from differences in post-translational modifications, such as glycosylation.

1.6.4. FGF Receptor Variants

Alternative splicing gives rise to a number of FGFR variants (Burgess and Maciag, 1989; Jaye *et al.*, 1992). The major *flg* variants include: deletions or insertions in the extracellular membrane domain; deletions in the cytoplasmic domain; *flg* variants encoding a secreted form; or, intracellular domain variants. A two-Ig form of *flg* has been found in a variety of tissues and species. One of the *flg* variants contains a VT-dipeptide deletion in the juxtamembrane region. This VT dipeptide deletion is also found in *bek* and FGFR-4 (Jaye *et al.*, 1992). Potential secreted forms FGFR-*flg* which possess either one or three Ig-like domains have been reported. Those secreted *flg* variants do not have the cytoplasmic portion of normal *flg*, but they have 31 irrelevant amino acid residues immediately downstream of Ig-like domain (Eisemann *et al.*, 1991). It has been shown that the ligand-binding specificity is located on the

second exon half of third Ig-like domain (Werner *et al.*, 1991). Intracellular domain variants includes insertion in the tyrosine kinase domain and truncated form of tyrosine kinase domain. Similar variants have also found in bek. Some of *flg* and *bek* variants involved use of alternative exons (Jaye *et al.*, 1992).

1.6.5. FGF Receptors in *Xenopus laevis*

Xenopus FGFRs have similar structure to FGFRs from other species. So far, cDNA representing two different FGFR genes have been cloned from *Xenopus* (Musci *et al.*, 1990; Friesel *et al.*; 1991; Friesel and Brown, 1992). They represent the *Xenopus* homologue of FGFR1(*flg*) and FGFR2 (*bek*). FGFR1 is expressed throughout all developmental stages and FGFR2 only expressed after post-gastrulation (Ruta *et al.*, 1988; Dionne *et al.*, 1990). One of the *Xenopus* FGFR1s (designated XFGFR-A1) was cloned from a cDNA library constructed from the *Xenopus* XTC cell line. (Friesel *et al.*, 1991). The other one (XFGFR-A2) was isolated from a *Xenopus* oocyte cDNA library (Musci *et al.*, 1990). The two XFGFRs are 95% homologous. Friesel *et al.*, also isolated another XFGFR-A2 variant which is identical to the sequence Musci *et al.*, cloned except for an 88 amino acid deletion at N-terminus.

There are evidence suggest that FGF is involved in mesoderm induction in *Xenopus* embryonic development (Slack et al, 1987; Gillespie et al, 1989; Amaya et al, 1991; Ryan and Gillespie, Submitted). It has also been reported that different concentrations of FGF can induce different mesoderm tissues (Smith and Slack 1983; Slack. et al., 1984 ; Slack et al, 1987; Gillespie et al, 1989; Kimelman. et al., 1992). Therefore, it is very important to understand the different responses mediated by FGF/FGFR signaling system during the mesoderm induction process. There are several mechanisms which FGF/FGFR signaling system might use to generate different intracellular signals. One of those is that FGF/FGFR signaling system can generate different intracellular signals by regulating the availability of various members of the FGFR family in different cells or embryonic tissues (Patstone et al., 1993).

So far, all *Xenopus* FGFR were cloned from oocytes or cell lines. No FGFR has been cloned from *Xenopus* embryos. The interest of this thesis work is to isolate and analyze FGFR cDNA from the developmental stage when mesoderm induction occurred. The experiments presented here were designed for cloning and characterization of one XFGFR-1 variant, XFGFR-A3, isolated from *Xenopus* embryos.

Chapter 2

Materials and Methods

2.1. Materials

Restriction endonucleases were purchased from Bethesda Research Laboratories (GIBCO-BRL, Life Technology, Inc. Burlington. ON. Canada), New England Biolabs, Inc. (Beverly, MA. USA), or Boehringer Mannheim Canada (Laval, Quebec).

T4 DNA ligase, RNase A, RNase T1, calf intestinal alkaline phosphatase (CIAP) and reverse transcriptase were purchased from GIBCO-BRL or Pharmacia Fine Chemicals (Piscataway, NJ. USA). Buffers for above enzymes were supplied by the manufacturer. The purified PKC was from Upstate Biotechnology Incorporated (Lake Placid, NY. USA.).

The *Xenopus* recombinant bFGF was expressed in E.coli and purified by heparin-Sepharose chromatography as described in Kimelman et al (1988).

Kodak XAR-5 X-ray film was purchased from Eastman Kodak Co. (Rochester, NY. USA).

The radioactive [α - 32 P]UTP, [α - 35 S]dATP, [α - 32 P]dATP and [γ - 32 P]dATP were purchased from Amersham Canada Limited (Oakville, ON. Canada).

Electrophoresis grade agarose was from GIBCO-BRL.

Acrylamide, bisacrylamide, TEMED, and ammonium persulphate were products of Bio-Rad Laboratories (Richmond, CA. USA.).

The pCDNAI neo-plasmid containing XFGFR-A2 clone was kindly provided by Dr. R. Friesel. The KS(+) plasmid containing the ODC fragment was a gift from Dr. J.M.W. Slack.

Bacto-tryptone, bacto-agar and yeast extract were bought from BDH. LB, NZY media, and LB/Ampicillin were prepared as described in Maniatis *et al.*, (1989). Ampicillin and tetracycline were purchased from Sigma Chemical Co., St.Louis, MO, USA.

The E.coli strains SURE and XL-BLUE were from Stratagene.

La Jolla, CA . USA.

2.2. Methods

2.2.1. Embryos, Dissections and Induction Assays

Xenopus embryos were obtained by artificial fertilization, handled and dissected as previously described by Godsave *et al.*, (1988) and staged according to Nieuwkoop and Faber (1975). Female *Xenopus laevis* were induced to lay eggs by subcutaneous injection of 500 I.U. of human chorionic gonadotrophin in 0.5 ml dH₂O. The eggs subsequently obtained were fertilized using ground *Xenopus* testes. Following rotation of the eggs, the jelly coats were removed using 2.5 % w/v cysteine hydrochloride (Sigma Chemical Co. St Lous, MO. USA.) adjusted to pH 7.8-8.1 with NaOH. They were then washed thoroughly and allowed to develop in petri dishes coated with 1.5 % agar (Difco, Detroit, MI. USA.) in 1/20 normal amphibian medium (NAM, Table 3; Slack and Forman, 1980).

Table 3. Composition of the NAM (Normal Amphibian Medium) used in the experiment.

10X NAM salts

	g/l (in 10X stock)	mM (Final in 1X)
NaCl	65	110
KCl	1.5	2
Ca(NO ₃) ₂ ·4H ₂ O	2.4	1
MgSO ₄ ·7H ₂ O	2.5	1
EDTA (0.5M, pH8)	2mls	0.1
Hepes (1M, pH7.5)	100mls	10

1X Solutions

For 100 mls of solution, add:

	NAM	NAM/2	NAM/20
10X NAM salts	10 mls	5 mls	0.5 mls
Gentamycin	0.25	0.25	0.25
Na Bicarb.	1	1	----
Sterile H ₂ O	88.75	93.75	99.25

2.2.2. Total RNA Isolation

RNA was prepared from whole embryos or animal cap explants by the LiCl/Urea method. This extraction method selectively precipitates RNAs with a high concentration of LiCl while small RNAs and DNA remain soluble. Proteins are denatured and solubilized by the presence of 6 M Urea. To 100 embryos, 2.5 ml of extraction buffer (3 M LiCl, 6 M Urea, 10 mM NaOAc pH 7.5, 0.1 % SDS, 0.5 % 2-mercaptoethanol) was added. The embryos were then homogenized and sonicated to shear DNA. The mixture was left on ice overnight to selectively precipitate RNA. RNA was pelleted by centrifugation of the overnight mixture at 12,000 g for 30 minutes. The RNA pellet was resuspended in 0.3M NaOAc pH 7.5 containing 0.5 % SDS and extracted with phenol/chloroform/isoamyl alcohol (1 : 1 : 0.02). After ethanol precipitation, the concentration of the RNA was determined by measurement of absorbance at 260 nm and 280 nm. The integrity of the RNA was verified by electrophoresis on a 1 % agarose gel containing 1 M formaldehyde buffered with 50 mM HEPES/EDTA pH 6.5. Total RNAs prepared by this method are good for mRNA purification, RT-PCR, and RNase protection assays.

2.2.3. mRNA Isolation

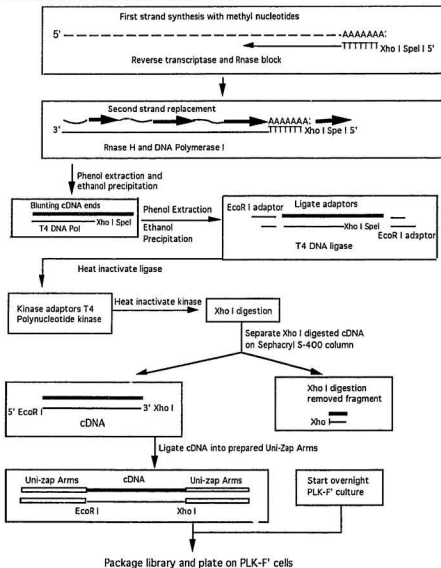
The quality and quantity of the mRNA used is of fundamental importance to the construction of a large, representative cDNA library. The PolyAtract mRNA isolation kit (Promega FisherScientific, Toronto, ON, Canada) was used to quickly produce a large quantity of undegraded RNA. mRNA isolation was performed according to the instructions provided with the PolyAtract kit. It is based on the magnetic separation of mRNA bound to oligo-dT particles from the total RNA population. Total RNA was dissolved in RNase-free water to give the desired concentration and annealed to biotinylated-oligo(dT) at 65°C in annealing buffer (0.5xSSC). Streptavidin paramagnetic particles (SA-PMPs) were washed with 0.5xSSC added to the annealing mixture and then incubated at room temperature for 10 minutes to allow the streptavidin to bind the biotinylated complex. The SA-PMPs-biotinylated-oligo(dT) mRNA complex was pelleted using the magnetic rack and the supernatant was carefully removed and discarded. The mRNA-containing particles were washed with 0.1xSSC and the captured mRNA was released by resuspending the final SA-PMP pellet in RNase-free water. The mRNA prepared in this way was of the high quality necessary for cDNA library construction.

2.2.4. Construction of cDNA Library

2.2.4.1. General Information

A cDNA library was constructed with mRNAs from stage 8 *Xenopus* embryos and a λ -ZAP cDNA Synthesis Kit (Stratagene, La Jolla, CA. USA). An outline of Stratagene's ZAP cDNA synthesis kit is provided (Figure 5). A detailed description of the steps involved in the construction of the cDNA library is provided in the following sections. Briefly, first strand cDNA was synthesized from 2 μ g of mRNA from stage 8 embryos with oligo(dT)-adaptors containing XhoI and SpeI restriction sites. 5-methyl dCTP was incorporated into the second strand to ensure that the restriction sites in the linker-primer were susceptible to restriction digestion. The uneven termini of the double-stranded cDNA was nibbled back or filled in with T4 DNA polymerase and EcoRI adaptors were ligated to the blunt ends. After XhoI digestion, the cDNAs were directionally inserted into λ Uni-ZAP XR vector arms (Stratagene) and packaged into a high-efficiency system, the Gigapack II Gold packaging extract. The packaged products were plated on SURE E.coli (mcrA- and mcrB- strain) to give the primary cDNA library (unamplified library). The first

Figure 5. The cDNA library construction chart (From Stratagene cDNA Libraries Construction Kit Chart).



amplified cDNA library was obtained by plating the primary cDNA library on the XL-Blue E.coli strain.

2.2.4.2. First Strand Synthesis

First strand cDNA synthesis begins when reverse transcriptase, in the presence of nucleotides and the appropriate buffer, finds a template and primer. The template is messenger RNA and the primer is a fifty base oligo consisting of "GAGA" protection sequences, XhoI and SpeI restriction enzyme recognition sites, and an 18 base poly (dT) sequence (Table 4).

In an RNase-free microcentrifuge tube, the following reagents were added: 5.0 μ l 10x first strand reaction buffer, 3.0 μ l 10 mM first strand reaction methyl nucleotide mixture, 2.0 μ l linker-primer (1.4 μ g/ μ l), 30 μ l DEPC-treated water, and 1.0 μ l RNase Block II (1 u/ μ l). The reagents were vortexed to ensure all the components are well mixed. Then 5 μ g mRNA from stage 8 *Xenopus* embryos (in 6.5 μ l DEPC-treated water) was added. The template and primer were allowed to anneal for 10 minutes at room temperature. Then 2.5 μ l of M-MVRT was added. The sample was gently vortexed and spun down in a microcentrifuge. 5 μ l of the mixture was transferred

Table 4. Sequences of polylinker and primer used in the cDNA library construction.

Name	Sequence
Polylinker	5'AATTCGGCACGAG 3' 3'GCCGTGCTC 5'
Primer	5'GAGAGAGAGAGAGAGAGAGAGAACTAGT CTCGAGTTTTTTTTTTTTTTTTTTT 3'

Figure 4. Autoradiograph showing the synthesis of high quality first and second strand cDNA from mRNA of stage 8 *Xenopus* embryos by M-MLVRT. The cDNA were used for cDNA library construction. Lane 1 and 2 show the parallel loading of the first strand cDNA product. Lane 3 and 4 show the parallel loading of second strand cDNA synthesis. Samples were separated by alkaline agarose gel electrophoresis(See methods section for details).



into a separate tube containing 0.5 μ l of the [α - 32 P]dNTP (800 Ci/mmol). This 5 μ l of first strand radioactive control sample allowed the analysis of the quality and quantity of the first strand synthesis. Both mixtures were incubated at 37°C for 1 hour. After 1 hour incubation, The first strand synthesis reaction mixtures were removed from the 37°C bath and stored at -70°C. The radioactive first strand control was later used for analysis of first strand synthesis (Figure 4.).

2.2.4.3. Second Strand Synthesis

In the 45 μ l first strand reaction mixture, the following reagents were added: 40 μ l 10x second strand reaction buffer, 6 μ l 10 mM second strand reaction nucleotide mixture, 300 μ l dH₂O and 2 μ l [α - 32 P]dNTP (800 Ci/mmol). The sample was briefly vortexed and the following enzymes was added: 0.9 μ l RNase H (4 U/ μ l) and 6.7 μ l DNA polymerase I (10.6 U/ μ l). The tube was quickly vortexed and incubated for 2.5 hours at 16°C. The tube was placed on ice immediately after synthesis. The mixture was extracted with an equal volume of phenol/chloroform and the aqueous layer was re-extracted with chloroform. The upper aqueous layer was transferred into a new tube. cDNAs were precipitated by adding 34 μ l of 3M

sodium acetate and 880 μ l of 100 % ethanol to the reaction mixture. The ethanol precipitation was spun down, and pellet was gently washed with 1 ml of 80 % ethanol. After washing, the pellet was resuspended in 43.5 μ l of dH₂O. 4.5 μ l was removed and frozen at -20°C for later analysis of second strand synthesis quality (Figure 4).

2.2.4.4. Blunting the cDNA Termini

In the tube containing 39 μ l second strand reaction mixture, the following reagents were added: 5 μ l 10xT4 DNA polymerase buffer, 2.5 μ l 2.5 mM dNTP mix and 3.5 μ l T4 DNA polymerase (2.9 u/ μ l). The tube was incubated at 37°C for 30 minutes. Then 50 μ l dH₂O was added to bring the volume up to 100 μ l. The sample was extracted with an equal volume of phenol/chloroform followed by re-extraction with an equal volume of chloroform. The aqueous phase was transfer into a new tube and precipitated by adding 7 μ l of 3M sodium acetate and 226 μ l of 100 % ethanol. The ethanol precipitation was spun down. Pellet was washed with 150 μ l 80 % ethanol.

2.2.4.5. Ligating EcoR I Adaptors

The pellet was resuspended in 7 μ l EcoR I adaptors followed by adding the following reagents: 1 μ l 10x ligase buffer, 1 μ l 10 mM ATP, and 1 μ l T4 DNA ligase. The reaction mixture was incubated overnight at 8°C. The ligase was inactivated by placing the tubes in a 70°C water bath for 30 minutes.

2.2.4.6. Kinasing the EcoR I End and Xho I Digestion

The following reagents were added into the tube containing the heat inactivated ligation mixture: 1 μ l 10x ligation buffer, 2 μ l 10 mM ATP, 6 μ l dH₂O, and 1 μ l T4 polynucleotide kinase (10 U/ μ l). The tube was incubated for 30 minutes at 37°C. The kinase was inactivated by heating for 30 minutes at 70°C. The reaction mixture was cooled to room temperature. 28 μ l Xho I buffer supplement and 3 μ l Xho I (45 U/ μ l) were added. The tube was incubated for 1.5 hours at 37°C followed by adding 5 μ l 10x STE buffer. The sample was loaded onto a ready-to-go Sephacryl S-400 Column and was spun in a table top centrifuge for 3 minutes at 700xg. The first fraction which contains large cDNAs was collected. 60 μ l of 1x STE was loaded onto the column and was spun again to get second cDNA fraction. The pooled cDNA fractions were extracted with an equal volume of phenol/chloroform. The upper aqueous layer

was transferred into a new tube and re-extracted followed by precipitating the cDNA from the aqueous layer with 2x volume ethanol. The cDNA was resuspended in 10 μ l dH_2O .

2.2.4.7. Ligating cDNA into Vector Arms

The following reagents were added into the tube containing 2.5 μ l of resuspended cDNA: 0.5 μ l 10x ligation buffer, 0.5 μ l 10 mM rATP, 1.0 μ l Uni-ZAP XR vector (1 $\mu\text{g}/\mu\text{l}$), and 0.5 μ l T4 DNA ligase (4 Weiss U/ μ l). The tube was incubated three days at 4°C followed by 2 hours at room temperature.

2.2.4.8. Packaging Instructions

1 μ l of ligation mixture was packaged into Gigapack II Gold packaging extract according to the packaging instructions. 1 μ l of ligation mixture and 15 μ l sonic extract were added to the tube containing quickly thawed packaging extract. The tube was stirred and spun quickly followed by incubation at room temperature for 2 hours. 500 μ l phage dilution buffer and 20 μ l chloroform were added. The tube was mixed gently and spun briefly to sediment debris. The supernatant was stored at 4°C and ready to be titered.

2.2.4.9. Plating and Amplification of Uni-ZAP XR Library

1 μ l of the diluted (1:10 dilution with SM buffer) and 1 μ l of the undiluted library were plated with 200 μ l of $OD_{600}=0.5$ SURE cells separately. Phage and bacteria were preincubated for 20 minutes at 37°C. 3 ml of top agar (48°C) was added and plated immediately onto prewarmed (37°C) NZY agar plates. Plates were incubated overnight at 37°C. The titer of this primary cDNA library was determined by counting the plaques formed on plates. To amplify the primary cDNA library, approximately 50,000 recombinant bacteriophage was added into 600 μ l of XL-BLUE cells adjusted to an $OD_{600}=0.5$. The cells were incubated at 37°C for 15 minutes. The cells were then mixed with 6.5 ml of melted 48°C top agar and poured evenly onto a prewarmed NZY 150 mm plate of bottom agar. The plates were incubated at 37°C for 6-9 hours. 10 ml of SM buffer was added onto each plate. The plates were incubated at 4°C overnight with gently shaking or rocking. The bacteriophage suspension was recovered from each plate and pool into a sterile polypropylene or glass container. The plates were rinsed with an additional 2 ml of SM and pooled. Chloroform was added to the pooled suspension to 5 % followed

by gently mixing. Cell debris was removed by centrifuging the suspension at 4000 g for 5 minutes. The supernatant was recovered, pooled and transferred to a sterile polypropylene or a glass bottle. Chloroform was added to 0.3 % and the library was stored in aliquots at 4°C. The titer of the primary amplified library was checked by using XL-BLUE cells and serial dilutions of the library.

2.2.5. Library Screening and cDNA Clone

Isolation

A 400 bp DNA fragment corresponding to part of the tyrosine kinase domain of the *Xenopus* FGFR (isolated by Gillespie, Figure 9B) was used to screen the cDNA library at low stringency. The 400bp fragment was gel purified and labelled with a random primer labelling kit (BRL) and [α -³²P]ATP (>400 Ci/mmol: Amersham). The library was prepared for screening as described by Kimmel A.R. (1987). Duplicate nitrocellulose membranes were used. The membrane preparation, probe preparation and screening protocol was from Wahl and Berger (1987). The probe was hybridized to the nitrocellulose membranes in 5x SSC, 2x Denhart's reagent, 200 ug/ml sonicated and denatured salmon sperm DNA, and 0.1% SDS

at 64°C for overnight and washed 2x 15 min in 1x SSC, 0.1 % SDS at 45°C; 2x 15 min in 0.5x SSC, 0.1% SDS at 45°C. Finally, the membranes were washed in 0.1x SSC, 0.1% SDS at 45°C for 20 min. Membranes were dried and exposed to Kodak XAR-5 film at -70°C with a Dupont Cronex intensifying screen. About 5×10^5 recombinant phages were screened. Positive clones obtained after secondary screening were purified according to Maniatis *et al.* (1989). Plaques of interested were cut out of from the agar plate and transferred into a sterile microfuge tube containing 500 μ l of SM buffer and 20 μ l of chloroform. The tube was vortexed to release the Uni-ZAP XR phage particles into the SM buffer and incubated 1 to 2 hours at room temperature or overnight at 4°C. cDNA inserts were rescued into plasmid form in pBluescript by *in vivo* excision using helper phage R408 as instructed in the Uni-Zap kit. 200 μ l of Uni-ZAP XR phage stock (containing $> 1 \times 10^5$ phage particles) was mixed with 200 μ l of $OD_{600}=1.0$ XL-BLUE cells and 1 μ l of R408 helper phage ($> 1 \times 10^6$ pfu/ml) in a tube and incubated at 37°C for 15 minutes. 5 ml of 2x YT media was added and the tube was incubated for 3 hours at 37°C with shaking. The tube was then heated at 70°C for 20 minutes, and spun for 5 minutes at 4000 g. The supernatant which contains the pBluescript phagemid packaged as filamentous phage particles was saved in a sterile tube. A serial dilution of this supernatant was

performed in order to plate the rescued phagemid. 200 μ l of each dilution was mixed with 200 μ l OD₆₀₀=1.0 XL-BLUE cells and incubated at 37°C for 15 minutes. 1 μ l and 100 μ l each was plated on LB/Ampicillin plates and incubated overnight at 37°C. Colonies appearing on the plate have the pBluescript double stranded phagemid with the cloned DNA insert. The plasmid was purified according to Maniatis et al., (1989) as described in Methods 2.11.

2.2.6. Sequencing Reaction

Double-stranded DNA was used for sequence analysis. Nucleotide sequencing was done by the dideoxynucleotide chain termination method (Sanger et al., 1977) using the sequenase V.2 kit (United States Biochemicals Corporation, Cleveland, OH. U.S.A.) and [α -³⁵S]dATP (>800 Ci/mmol. Amersham) on double-stranded plasmid DNA templates according to the instructions provided. 2 μ l of each dNTP sequencing reaction sample were loaded on 1xTBE buffered 6% acrylamide sequencing gels. The gel was run at 55 W for 2-3 hours then was transferred onto Whatman 3MM paper and dried at 75°C. The gel was exposed to Kodak XAR-5 X-ray film (Eastman Kodak Co., Rochester, NY, USA.) overnight with an intensifying

screen.

Both strands of the XFGFR-A3 were sequenced with synthesized oligonucleotide primers (Oligos, Etc.), and the sequences were saved and analyzed using the PC gene program(Intelligenetics).

2.2.7. Sequencing PCR Products

Modified dideoxynucleotide chain termination method was used to sequence PCR products directly with the Sequenase V.2 kit. Around 80ng primer was mixed with 200-800 ng purified PCR products in 6 μ l of 40 mM Tris-chloride pH 7.5, 25 mM $MgCl_2$, 50 mM NaCl, 10% DMSO. After boiling for three minutes to denature the template, the mixture was immediately cooled on dry ice to minimize template renaturation. 4 μ l of labelling mix containing 25 mM DTT, 10 μ Ci [α - 32 S]dATP (>800 Ci/mmol. Amersham), and 2 units of Sequenase (USB) was added. The resulting 10 μ l mixture was divided equally into four tubes each containing 2 μ l of 80 μ M dCTP, dGTP, dTTP, dATP, 50 mM NaCl, 10% DMSO and 0.08 μ M ddATP (tube A), 8 μ M ddCTP (tube C), 8 μ M ddGTP (tube G) or 8 μ M ddTTP (tube T). The tubes were incubated at 37°C for 2-5 minutes, then 4

μ l of stop buffer was added into each tube. The samples were heated to 75° c for 3 minutes before loading onto sequencing gel.

2.2.8. RT-PCR Analysis of Gene Expression

2.2.8.1. Reverse Transcription Reaction

A standard reverse transcription protocol was used (as described by Maniatis et al., 1989). 20 ug of total RNA prepared from different stage *Xenopus* embryos was reverse transcribed into cDNA in 40 μ l reaction volume with M-MLVRT (Moloney-Murine Leukemin Virus Reverse Transcriptase) by using a oligo(dT) primer. Total RNA from stage 2, stage 6, stage 8, stage 12, stage 16, stage 24, stage 30 and stage 41 *Xenopus* embryos were used (Figure 10A).

2.2.8.2. PCR Methods

RT-PCR analysis was performed according to the RT-PCR methods described by Erlich (1989). The PCR reaction was done in a 50 μ l volume and, in addition to the sample DNA, contained 50 mM KCl, 10 mM Tris.HCl (pH 8.4), 1.5 mM MgCl₂, 100 μ g/ml gelatin, 0.25 μ M of each primer, 200 μ M of

deoxynucleotide triphosphate (dATP, dCTP, dGTP. and dTTP), and 2.5 units of *Taq* polymerase. A few drops of mineral oil was often added to seal the reaction and prevent evaporation. The amplification was performed in a Perkin-Elmer/Cetus Thermal Cycler (Perkin-Elmer Cetus Instruments) using the program set to denature at 94°C for 1min, anneal at 58°C for 50 sec, and extend at 72°C for 58 sec for a total of 30 cycles. These conditions were used to amplify the XFGFR1 genomic DNA fragment flanked by T305 and S402 primers. Modified conditions were used to perform RT-PCR reactions. The first cycle of RT-PCR reaction was performed by oligo(dT)-adaptor with longer chain extension time to synthesize full length first strand cDNA from total RNA template. Starting from second PCR thermal cycle, genomic DNA PCR conditions were used (Figure 14A).

2.2.9. Synthesis of RNA Probes with T3 and T7

RNA Polymerase

In vitro transcription was used to generate RNA probes for RNase protection assays (Ausubel et al., 1993). *In vitro* transcription by T3 or T7 RNA polymerase was carried out in 40 mM Tris, pH 7.5, 6 mM MgCl₂, 2 mM spermidine, 10 mM DTT, 2

units/ μ l RNase Guard (Pharmacia), 100 μ g/ml BSA, 500 μ M of each rNTP and 100 μ g/ml of linearized plasmid DNA. The *in vitro* transcription reaction was allowed to continue for 30 minutes at room temperature. Then 0.5 unit/ μ l RNase-free DNase was added to remove the DNA templates. The reaction mixture was then passed through a G-50 column to separate the synthesized RNA probe from unreacted (α - 32 P)UTP. After column purification, the *in vitro* transcription products were subjected to phenol/chloroform extraction and ethanol precipitation in the presence of 1 μ l of 10 mg/ml yeast tRNA.

The probe was further purified by separation through a 6% native PAGE gel. After running the gel, a short exposure of the gel on X-ray Kodak X-ARP film was taken (a few min). The gel slice containing full-length probe was excised from the gel with the help of the exposed film. Full-length labelled probes were eluted by incubating the gel slice with 2 M ammonium acetate, 1% SDS, and 25 μ g/ml tRNA at 37°C for 2-4 hours with shaking. Probes purified by this method gave the best results for RNase protection assays.

2.2.10. RNase Protection Assays

RNase protection assays were performed according to the ribonuclease protection assay protocol described by Ausubel et al., (1993). Full-length probes were purified by gel electrophoresis followed by ethanol precipitation in the presence of 10 μ g yeast tRNA. 10 μ g of total RNA from different stage *Xenopus* embryos were hybridized at 45°C overnight with 10⁶ cpm of the labelled antisense probe in a solution containing 50 % formamide, 40 mM PIPES (PH 6.7), 400 mM NaCl, and 1 mM EDTA. The mixture was digested with RNase T1 for 60 mins at 33°C followed by phenol/chloroform extraction and ethanol precipitation. The protected products were separated on 6 % polyacrylamide-8 M urea gel. As a negative control, antisense probe was hybridized to 10 μ g of tRNA. The positive control contains the 260 bp XFGFR-A2 probe hybridized to XFGFR-A2 cRNA prepared by *in vitro* transcription.

2.2.11. Large Scale Preparation of Plasmid DNA

An overnight culture of the E.coli strain containing the required plasmid in 20 ml LB Amp (50 μ g/ml ampicillin) was incubated at 37°C with vigorous shaking (250 RPM). 5 ml of

the overnight culture was added to 500 ml of fresh LB Amp media. The cells were allowed to grow until the cell density reached OD₆₀₀ of 1.0. To get a high copy number of plasmid per bacterium, 1 ml of chloramphenicol (1 mg/ml in ethanol) was added and cells were allowed to grow for a further 12-16 hours. Cells were harvested by centrifugation at 4000 g for 5 minutes. The plasmid was isolated from the bacteria pellet by alkali lysis method (Maniatis et al., 1989). The crude plasmid was further purified by cesium chloride density gradient ultracentrifugation methods as described by Maniatis et al., (1989). Briefly, the crude plasmid DNA was mixed with ethidium bromide and cesium chloride and subjected to equilibrium centrifugation at 45000 g for 16 hours. The rotor was allowed to decelerate freely. The supercoiled plasmid band was removed from the Beckman ultracentrifugation tube by puncturing the side of the tube with a 18 or 21 gauge needle and withdrawing the supercoiled plasmid solution into a syringe. The supercoiled plasmid solution was extracted with water-saturated butanol to remove the ethidium bromide. The plasmid solution then was diluted with two volumes of dH₂O and subjected to ethanol precipitation. The purity of the plasmid was checked on a 1% agarose gel.

2.2.12. Subcloning Manipulation

2.2.12.1. Restriction Endonuclease Digestion of Plasmid DNA

Restriction endonucleases were used to manipulate plasmid. The recognition sequences, the cleavage sites, and the sources of the restriction endonucleases used in this research work are given in Table 6.

The digestion temperatures, times, buffers, reaction volume, as well as restriction enzyme and sample DNA amount used were all according to the directions given by the individual restriction enzyme manufacturer.

2.2.12.2. Agarose Gel Electrophoresis to Separate DNA Fragments

To get the desired DNA fragments after restriction endonuclease digestion, the digested plasmid DNAs were separated on 1xTBE buffered 0.8-2% agarose gel. The gel was stained in 1 µg/ml ethidium bromide for 20-30 minutes after electrophoresis. The DNA was visualized using an ultra-violet (UV) transilluminator (Hoeffer Scientific Instruments. San

Francisco, CA. USA.) or a portable longwave UV lamp (Fotodyne. San Francisco, CA. USA.). The gel was photographed with a Polaroid MP-4 camera and Kodak 22 A filter or by using a photodocumentation system (Stratagene, La Jolla, CA, USA.). The gel slice containing the target DNA fragment was cut out of the gel, put into dialysis tubing, and subjected to electroelution. After electroelution, the DNA fragments were recovered and purified by phenol/chloroform extraction and ethanol precipitation of the solution inside the dialysis tubing.

2.2.12.3. Blunt End Ligation

Most restriction endonucleases give staggered cuts on DNA molecules. There are two ways to blunt the staggered ends. T4 DNA polymerase can cut back or fill-in the sticky DNA ends to give even ends. Klenow fragment (E.coli. DNA polymerase I large fragment) can fill in the 3' recessed staged ends.

Because restriction endonucleases leave the 5' phosphate intact, the Klenow filled in product or blunt-cut restriction endonuclease digestion product can be used directly in the blunt end ligation reaction without phosphorylation.

The subcloning vector for blunt end ligation must be cut in the polylinker region, blunted, and dephosphorylated on both ends. In our case, EcoR V was used to cut KS(+) Bluescript plasmid (Stratagene) to yield blunt ends and calf intestinal alkaline phosphatase (CIAP) was used to remove the 5' phosphate group.

Sample DNA fragments with blunt ends were mixed with prepared vectors in the proper ratio (Maniatis et al., 1989) in a final volume of 10-20 μ l of 1x T4 ligation buffer containing 25% PEG 6000 (polyethylene glycerol), 1 mM ATP, and 2 units of T4 ligase. The ligation reaction mixture was allowed to incubate overnight at 12-14 $^{\circ}$ C. The ligation mixture can then be used to transform bacteria.

2.2.12.4. Preparation of Competent Cells by the Calcium Chloride Method

A culture was grown from a single bacterial colony at 37 $^{\circ}$ C in 500 ml of LB broth with vigorous shaking until the OD₆₀₀ reached 0.5-1.0 (depending on the E.coli strain used). The cells were chilled by placing on ice for 30 minutes and pelleted by centrifugation at 4000 g for 15 minutes at 4 $^{\circ}$ C. The cell pellet was resuspend in 200 ml of ice cold filter-

sterilized 100 mM CaCl₂ and centrifuged to pellet the cells. This wash was repeated twice and the cells were resuspended in a final volume of 5 ml filter sterilized 100 mM CaCl₂. The suspension was stored on ice for 20-24 hours. Cold, autoclaved, sterile glycerol was added slowly to the cell suspension to give a final concentration of 15%. The cell suspension was aliquoted, frozen immediately on dry ice and stored at -70°C.

2.2.12.5. Transformation of Competent E.coli

XL-Blue Cells

For transformation, 50 µl of freshly thawed competent cells were mixed with 2-10 µl of the blunt-end ligation mixture in a prechilled 1.5 ml eppendorf tube. The tube was put on ice for 1 hour and transferred into a 42°C water bath for a 1 minute heat shock. The tube was then placed back on ice for 2 minutes. 900 µl of 37°C LB media was added into the tube. The cells were allowed to grow at 37°C for 1 hour then plated onto LB/Amp plates and grown overnight at 37°C. Only transformed cells which carried the antibiotic gene will form colonies on the plates.

2.2.13. Construction and Analysis of GEX-FGFR(VT+) and GEX-FGFR(VT-) Fusion Proteins

2.2.13.1. Construction of the GEX-FGFR(VT+) and GEX-FGFR(VT-) Fusion Proteins

pGEX vectors can be used in bacterial systems to express foreign polypeptides as fusion products with glutathione S-transferase (GST) (Smith and Johnson, 1988). Each pGEX-KT (Kinker-Thrombin) vector contains an open reading frame encoding GST, followed by unique restriction endonuclease sites for BamHI, SmaI, and EcoRI, followed by termination codons in all three frames.

The pGEX-FGFR(VT+) expression vector was obtained by subcloning the 144 bp DraI-BsaHI cDNA fragment from XFGFR-A2 into the EcoRV site of pGEX-KT by blunt-end ligation. This subclone was designed to leave only one threonine residue (Thr⁴²⁴) in the FGFR portion. The pGEX-FGFR(VT-) expression vector was obtained by using the same subcloning strategy; but there was a 74 bp sequence introduced following the 138bp DraI-BsaHI cDNA fragment from XFGFR-A3 in the pGEX vector (Figure 6). This 74 bp fragment introduced two threonine

Figure 6 B. Deduced amino acid sequence of the GEX-FGFR(VT+) and GEX-FGFR(VT-) cloning region and FGFR insertion. The GEX-KT vector sequence is undrelined and FGFR fragment sequence is shown in bold.

GEX-FGFR(VT-) sequence

5' PKSDLGGGGGLVPRGSPGKMKHPSKKSDFNSQLAVHKLAKSIPLRRQV
SGDSSSSMNSGVILVRRFKLIDKLDVTIIFNSS 3'

GEX-FGFR(VT+) sequence

5' PKSDLGGGGGLVPRGSPGKMKHPSKKSDFNSQLAVHKLAKSIPLRRQVTV
SGDSSSSMNSGVILVRRRI 3'

residues on the FGFR; but neither of these two threonine were contained within PKC phosphorylation site motifs (Graff et al., 1989) hence neither site should be phosphorylated by PKC. This pGEX-FGFR(VT-) provides a good control for the *in vitro* PKC assay.

2.2.13.2. Purification of GST-FGFR(VT+) and GST-FGFR(VT-) Fusion Polypeptides Expressed in E.coli.

After subcloning the chosen DNA fragment into the pGEX-KT vector in the correct reading frame, competent E.coli strain JM109 cells were transformed and transformants were selected on LB/Amp plates. Transformant colonies were picked after incubating these plates 12 to 15 hr at 37°C. These colonies were used to prepare plasmids. The picked colonies were sequenced to verify the constructions. The pGEX-FGFR(VT+) and pGEX-FGFR(VT-) were expected to yield 32 kD and 35 kD fusion protein respectively. The GST gene used to generate the pGEX-KT vector gives a 26 kD protein by itself. Large scale fusion protein purification was performed according to Smith and Corcoran (1993). Briefly, overnight cultures of E.coli transformed with parental or recombinant pGEX-KT plasmids were diluted 1:10 in 800 mls of fresh medium and grown for

about 1 hr before adding IPTG to 0.1 mM. After a further 3 hr of growth, cells were pelleted and resuspended in PBS at 1/50 of the culture volume. The cells were lysed on ice by mild sonication following the addition of 1% Triton X-100 (Bio-Rad). Extracts were then subjected to centrifugation at 10,000xg for 5 min at 4°C to remove insoluble material. The supernatant was mixed at room temperature in a 50-ml polypropylene tube on a rotating platform with 2.5 mls 50 % glutathione-agarose beads (Pharmacia). Beads were pre-swollen in PBS, washed twice in the same buffer and stored in PBS at 4°C as a 50 % solution (v/v). After a 2 min incubation period, beads were collected by brief centrifugation at 500xg and washed three times with 50 ml PBS. Fusion protein were eluted by competition with reduced glutathione (Boehringer Mannheim), using 2x2-min washes with 1 bed volume of 50 mM Tris.HCl (pH 8.0) containing 5 mM reduced glutathione (final pH 7.5, freshly prepared).

Contamination of purified fusion proteins with E.coli proteins, was reduced by including Triton X-100 during the incubation with glutathione-agarose and also by minimizing the period of sonication. The yield of unstable fusion proteins was increased by reducing the induction period to one hour. Yields of fusion protein were calculated from the

absorbance at 280 nm using the relation $1 A_{280} = 0.5 \text{ mg/ml}$ and using bovine serum albumin as a standard. Purified fusion proteins were analyzed on SDS-PAGE.

2.2.13.3. PKC Assay on GST-FGFR(VT+) and GST-FGFR(VT-) Fusion Polypeptides.

The purified fusion proteins were tested for their ability to act as substrates for PKC in vitro. The assay system (BRL) is designed to measure incorporation of $^{32}\text{P}_4$ into protein, catalyzed by purified PKC (UBI). The positive control for these experiments measures phosphorylation of a specific substrate for PKC (Yasuda et al., 1990); this substrate is a synthetic peptide corresponding to a.a. 1-14 of myelin basic protein. As a negative control, phosphorylation of each protein/peptide was also performed in the presence of a specific inhibitor of PKC activity. The inhibitor is a pseudosubstrate peptide corresponding to a.a. 19-36 of PKC (House and Kemp, 1987).

Each assay tube contained 15 ng PKC in 20mM Tris, pH 7.5, 20mM MgCl_2 , 1mM CaCl_2 , 20uM ATP, 10uM phorbol 12-myristate 13-

acetate (PMA), 280µg/ml phosphatidylserine in 0.03% Triton-X100, 5µCi α -³²PATP and either 50µM substrate peptide or 4µM fusion protein in a final volume of 50µl. The negative controls also contained 20µM inhibitor peptide. The enzyme was pre-incubated with or without the inhibitor on ice for 20 min, to allow the inhibitor to bind. After addition of the remaining components, the samples were incubated for 15 min at 30°C and 20µl aliquots were spotted onto phosphocellulose discs. The discs were washed twice with 500 mls of 1% phosphoric acid and twice with distilled water, placed in 10 mls Biodegradable Counting Scintillant (Amersham) and counted in a Beckman LS3801.

Chapter 3

Results

3.1. Stage 8 *Xenopus* cDNA Library Construction

There is evidence that FGF is involved in mesoderm induction in the *Xenopus* embryo. Our interest was to investigate intracellular signal transduction during mesoderm induction. This process begins with the binding of FGF to the FGFR. Characterization of FGFR genes that are expressed in early *Xenopus* embryos and how these FGFRs are regulated is an important aspect of the study of signal transduction. So far, all FGFRs cloned from *Xenopus* are from oocytes or a *Xenopus* cell line. No FGFR has been cloned from embryos. Therefore, given the diversity of identified FGFR gene products, it was important to isolate FGFR cDNA from the developmental stage when mesoderm induction is believed to occur in order to obtain a more accurate or precise concept of the molecular signalling events responsible for the first embryonic differentiative event. For this reason, I constructed a cDNA library from *Xenopus* stage 8 blastulae.

Two methods that are commonly used to make cDNA libraries differ in the manner that in which the cDNA is converted into a double-stranded form. The most popular method uses the "nick-translation" procedure developed by Gubler and Hoffman (1983). This method uses the mRNA template as a primer for second strand cDNA synthesis. Before the development of this method most cDNA libraries were made by the " hairpin-extension" method in which a hairpin structure at the 5' end of the first strand cDNA is used as a primer for synthesis of the second strand cDNA. The hairpin structure is later removed by S1 nuclease.

Sometimes it is very difficult to reverse transcribe the 5' end of the mRNA sequence into cDNA. This is because either the mRNA is very large or because the mRNA has secondary structures which prevent it from being reverse transcribed. Therefore, it is important to select for large cDNAs (>500 bp) before insertion into the construction vector (see cDNA library construction chart; Fig.5). cDNA was synthesized from poly (A) tail of the mRNA and cDNAs were fractionated on sephacryl S-400 column so only cDNAs >500 bp in size were used to construct the cDNA library.

A λ -ZAPII primary cDNA library was constructed with a size of 7×10^6 recombinants / μ g of λ phage arms. The primary amplified cDNA library had a titer of 5×10^{11} p.f.u./ml (plaque forming units/ml). Forty plaques from the primary amplified cDNA library were picked at random and checked by PCR with T3 and T7 primers for the proportion containing inserts and the range of insertion size. Thirty-eight colonies had insertions with an average size of 800 bp. The smallest one had 200 bp insertion and the largest has 5 kb insertion. We can estimate then that at least 95% of the recombinants had cDNA inserts. This result met the cDNA library construction criteria (over 90 % of the recombinants have to have cDNA inserts) The construction of this λ -ZAPII cDNA library from stage 8 *Xenopus* mRNAs was a successful one, considering 5×10^6 recombinant bacteriophages is a mammalian cDNA library of reasonable size. Only primary amplified cDNA libraries were used for the screening process to isolate XFGF receptor cDNA clones.

3.2. Isolation and Sequence Analysis of the Full Length XFGFR-A3 cDNA Clone

Approximately twenty positive plaques were isolated by low stringency screening of the primary amplified stage 8 *Xenopus* cDNA library with a 400 bp probe consisting of part of the tyrosine kinase domain of *Xenopus flg*. The 400 bp sequence had been cloned from PCR amplification of *Xenopus* stage 17 cDNA using primers designed from the chicken *flg* sequence. A phage density of 5000 p.f.u per 150 mm NZY plate was used in the first screening process. Each positive from this screen was a mixture of several overlapping phage plaques. All twenty positives were subjected to PCR amplification with FGFR specific primers. Only one positive named 8A-1, gave a positive PCR result. This positive was used for a second plating with lower phage density so that single plaque could be picked from the plate. By combining T3 or T7 primers with FGFR1 specific primers XFGFR5', XFGFR3', or Sequence 4 (Table 4), this particular cDNA clone was identified as a XFGFR1 cDNA clone according to PCR results (Data not shown). The predicted full-length XFGFR1 cDNA was 3.5-4 Kb based on previously cloned XFGFRs. This λ phage clone was then rescued into plasmid form with the help of the R408 phage.

Large scale plasmid preparation and restriction endonuclease analysis were carried out. This positive clone was sequenced using the dideoxynucleotide termination method with synthetic oligonucleotides. Seven batches of oligonucleotides were synthesized in order to get the complete sequences from both strands (Table 4). The sequencing data was stored and analyzed with the PCGENE program (Intelligenetics). This newly identified XFGFR1 variant was designated XFGFR-A3 to distinguish it from the two previously identified *Xenopus* FGFRs XFGFR-A1 and XFGFR-A2 (Friesel and Dawid, 1991). XFGFR-A3 cDNA is 3863 bp in length (Figure 7). It has a 182 bp 5' and 1250 bp 3' non-coding sequence. Its open reading frame (from nucleotides 183 to 2613) is 2430 bp in length. The open reading frame of XFGFR-A3 is predicted to encode a protein containing 810 amino acids (Figure 7). The predicted XFGFR-A3 protein sequence has virtually the same structure as previously reported FGFRs. It consists of a 21 aa signal peptide, a extracellular ligand binding domain (351 aa) with three Ig-like domains and an acidic box between first and second Ig domain, a transmembrane domain of 21 aa and a cytoplasmic domain of 417 aa. The cytoplasmic domain includes a 74 residue juxtamembrane domain and a tyrosine kinase domain split by a 14 aa insertion. The 1250 bp 3' non-coding region contains a potential cytoplasmic

Figure 7. Nucleotide sequence and deduced amino acid sequence of the XFGFR1-A3 cDNA clone. The presumed signal peptide sequence, transmembrane region, and the potential cytoplasmic polyadenylation element and the hexanucleotide polyadenylation signal AATAAA in the 3' end untranslated region are underlined.

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1  GGCACGAGTCTCGTAGATTTTTTTTTGAAAAATAATTTTCCCCCTACTAATAAAAGGAAT
61  ATAAGTGTGGACGATCCGAGATAAGAACTAAACATTGGAAACAGGAGCTGTGTGAGG
121 CTTGTCTCGTGCCCTGACACTGGAGGTCTCATGGATTTGGCTGTGTGCACTAGCCAAC TT
181 GGGATGTTCTCCGGAAGGTCCCTCCTCCTCTGGGGTGTCCTGCCTGGAGCTGCTTTTACA
    M F S G R S L L L L W G V L L G A A L S
241 GTT3GCCCGGCCCTTCCACCCTCCCGACCAAGTCGCCCCATAAAACCAAAACGGAAGTG
    V A R P P S T L P D Q V A P K T K T E V
301 GAGCCGTATTCTGCTCAGCCAGGAGACAGGATAACTTTGCAGTGCAGGCTACGAGAAGAT
    E P Y S A Q P G D R I T L Q C R L R E D
361 GTTCAGAGCATCAACTGGGTGAAAAATGGAGTTTCAGCTTTTCGGAGACTAACCGCACGCGC
    V Q S I N W V K N G V Q L S E T N R T R
421 ATAACAGGGAGGAGATCCAAATTTCCAACGCAGGGCCGGAGGACAATGGGGTGTATGCC
    I T G E E I Q I S N A G P E D N G V Y A
481 TGTGTTACTAACGGCCCTTCTCGAACATATACAGTATTATTCTCCGTTAATGTATCAGAT
    C V T N G P S R T Y T V L F S V N V S D
541 GCACTGCC3TCGGCCGAGGACGATGATGAAGATGATGACAATTCATCCTCTGAGGAGAAA
    A L P S A E D D D E D D D N S S S E E K
601 GCTGCAGAAAAC3TCCAAACCGAACCGTCCACTTTGGTCACATCCAGAAAAAATGGAGAAG
    A A E N S K P N R P L W S H P E K M E K
661 AAGCTTCATGCAGTGCAGCA3AAAAACTGTAAAAATTTAGGTGTCCAGCAAAATGGAACT
    K L H A V P A A K T V K F R C P A N G T
721 CCAACGCCAACACTTCGCTGGCTGAAGAAATGGCAGGGCGTTCCAACAAGATCAGCGCATT
    P T P T L R W L K N G R A F Q Q D Q R I
781 GGTGGATATAAGGTCCGGTCTCAACATGGAGTCTTATTATGGATTTCGGTGTGCCATCT
    G G Y K V K S Q T W S L I M D S V V P S

```

841 GATAAAGGAAATTACACCTGTATTGTGGAGAACAAGTATGGTGCATCAACCACACATAC
 D K G N Y T C I V E N K Y G A I N H T Y
 901 CAGTTA⁵GATG³TGTGAGCGTTCCCCACACGCCCAATCTTACAAAGCCGGTCTCCACGG
 Q L D V V E R S P H R P I L Q A G L P A
 961 AATACAAGCGTTACTGTGGGAACACAGCCGAATTTCTCTGCAAAGTGTAAGCGACCC
 N T S V T V G T T A E F S C K V Y S D P
 1021 CAGCCTCACATCCAATGGCTCAGGCACATTGAAATTAACGGCAGCAGAGTGGCCTCGGAT
 Q P H I Q W L R H I E I N G S R V A S D
 1081 GGCTTCCCGTATGTGGAGATCCTCAAGACTGCAGGAGTCAACACCTCGGACAAAGGATATG
 G F P Y V E I L K T A G V N T S D K D M
 1141 GAGGTCTCCACCTGAGAAATGTTACTTTTGGAGATGCTGCCAGTATACCTGCTTGGCC
 E V L H L R N V T F E D A G Q Y T C L A
 1201 GCTAACTCCATTGGGATCTCTCATCATTTCTGCATGGTTGACCGTTCTTAAAGTTGAGGAC
 A N S I G I S H H S A W L T V L K V E D
 1261 AATAAACCTGCGCTTCTGGCCTCCCTTTTACAAC⁵TGGAAATTAATCATCTACTGCACGGG
 N K P A L L A S P L Q L E I I I V C T G
 1321 GCTGCTTTTGTGTCGCAATGGTGGTCACCATCATTATCTTTAAATGAAGCACCCGTCG
A A F V S A M V V T I I I F K M K H P S
 1381 AAGAAGTCGGACTTCAACAGCCAACCTGGCTGTGCACAAGCTTGCAAGAGCATCCCGCTG
 K K S D F N S Q L A V H K L A K S I P L
 1441 CGCAGACAGGTTTCAGGGGACTCCAGCTCATCAATGAACTCGGAGTGATATTAGTCAGA
 R R Q V S G D S S S S M N S G V I L V R
 1501 CGCCTTTCTTCCAGTGGGACTCCCATGTTGTCTGGACTATCGGAATATGAGCTTCCAGAA
 R L S S S G T P M L S G L S E Y E L P E
 1561 GATCCACGATGGGAAGTGGCAAGGACAGACTGATCCTTGGGAAACCTCTCGGAGAAGG
 D P R W E V A R D R L I L G K P L G E G
 1621 TGCTTTGGGCAAGTAGTCAATGGCGGAGGCTATTGGCCTGGACAAAGGAGAAGCCTTAACAAA
 C F G Q V V M A E A I G L D K E K P H K
 1681 GTAACAAAAGTTGCTGTGAAGATGTTGAAGTCTGATGCGAGTGAAGGACCTGTCCGAT
 V T K V A V K M L K S D A S E K D L S D
 1741 CTGATTTCAGAGATGGAATGATGAAATGATTGGAAAACAAAAATATAATTAATTTA
 L I S E M E M M K M I G K H K N I I H L

1801 CTTGGTGCCTGCACCCAAAGATGGTCCACTCTATGTAATTGTGCAATACACTTCCAAGGG
 L G A C T Q D G P L Y V I V E Y T S K G
 1861 AATCTGAGAGAGTACTTACGGGCCAGGCGCCGCCGGATGGAGTACTGCTACAAACCT
 N L R E Y L R A R R P P A M E Y C Y N P
 1921 ACCTGTGTCCTCCGATCAGCTGCTTTCCTTCAAAGATCTGGTGTCAATGCTTACCAGGTG
 T C V P D Q L L S F K D L V S C A Y Q V
 1981 GCACGTGGGATGGACTACCTAGCCCTTAAAAAGTGCATCCACAGAGACCTGGCTGCAAGG
 A R G M D Y L A S K K C I H R D L A A R D
 2041 AATGTTTTAGTAACAGAGGACAACATAATGAAGATTGCCGATTTCCGCTTAGCCCGTGAC
 N V L V T E D N I M K I A D F A L A R D
 2101 ATCCATCACATGACTATTATAAGAAAACGACAAATGGCCGGCTGCCTGTAAAAATGGATG
 I H H I D Y Y K K T T N G R L P V K W M
 2161 GCCCCAGAAGCACTGTTTGGACCGATTACACTCATCAGAGCGATGTGTGGTCGTTTGGC
 A P E A L F D R I Y T H Q S D V W S F G
 2221 GTGCTGCTGTGGGAGATTTTCACACTGGTGGCTCCCCATATCCGGGTGTCCCCATGGAA
 V L L W E I F T L G G S P Y P G V P M E
 2281 GAACTCTTTAAGTTACTCAAGGAAGGGCACAGGATGGATAAACCCTAACTGCAACCAAT
 E L F K L L K E G H R M D K P T L C T N
 2341 GAGTTGTATATGATGATGAAGGACTGTTGGCATGCCATGCCCTCTCAAAGACCAACATTC
 E L Y M M M K D C W H A M P S Q R P T F
 2401 AATCAGCTGGTTGAGGATCTTGACCGAATCTTGCTCTGAGTTCCAATCAGGAATATCTT
 N Q L V E D L D R I L A L S S N Q E Y L
 2461 GATCTCTCCATGCCAGTGAATCAGTATTCTCCATGTTTCCAGACACTCGAAGTTCGAGG
 D L S M P V N Q Y S P C F P D T R S S L
 2521 TGTTCTTCAGGCGAGGACTCTGTGTTCTCTCATGACCCCCCTCCCGATGAGCCCTGCCTT
 C S S G E D S V F S H D P L P D E P C L
 2581 CCCAAATACTCCAATGGTGGACTTAAAAACGCTGACCTTGGGGGTGTTTGGGGTTC
 P K Y S N G G L K K R
 2641 TCTCCATLATTCCATTAAAGAACATTTGTTTTGAAGCATTCCAGAAATGCCACAAGTT
 2701 CCTCAGAAGCATGGTACAATTCCACTCCTACATCTTCAGCCTTATTCCTTTACACATGCA
 2761 GTTTGTAGGAGGTACTTTTTTGGTTGAGGATGGAACCAAGCAATATTCCTAATGTTGACC

2821 TCCAGCCAGTGCAC~~TTTCT~~AAAAATGCAACAGTGTCAAGATCCAAGGTCGCACCATGTG
 2881 ACATGGCAATATCATGGGAAC~~TTATCGAGTAGAAACTTTGCCAACTCTTTTAATATTCCT~~
 2941 CAATTTTATGAGATTTTATTTTCC~~TGACCTCATCTATATATCAAAACCTGACCGGTGTC~~
 3001 GAAACCTTCTCAAACACTCCAGGCTACCATTTAAGACCATCGTTTGGCTTTTCTGCA~~C~~
 3061 TGCTGAGCTCATCTTTTGGTGGAAAAATATTCATGGACAACACTCCTTCTCACAAC~~TC~~
 3121 CTTGTAGCTAGGTTAGACTTTCTGCTTGCTCTGAGGAAGCCGATTCCTCTCATATTC~~CA~~
 3181 ATGCAAGAGGTGCCTGCTTGAAC~~TGAATTATGAAGGGAGACCTCA~~TTATAAGCTACAT
 3241 ACCATCTCTGCTTCCTGAATGTTTATTATGCACGAGGTGGATTTTCAAAGTC~~TTAATT~~
 3301 AGCAAGTACCTGCATAAAGGCACAAGATATGGACTGTGCTCATATGCAACCTAAAA~~AAA~~
 3361 ATCATTTTATTTCAGGAATGTTAGTGATCTGTTAATGGTACGATCAATATG~~CTGTTGAC~~
 3421 TGCAACGTTTTTACTAGAGGTTTGACTGAAGGCGTGTGTTTCAGTGAAAGCCCATATA~~A~~
 3481 AGTCGGTAGAACTAGAGGCTTTCATCTCACTCCTAAGGTCCACATCAAAATGTCAGAAA~~A~~
 3541 ACATGGGAAATAGTCAAATTTTATCCTAATAAAAAATGGGGTGGCAGGGAAATCCAA~~TAT~~
 3601 GCTTTCCTTTTGTATATAGCTAAAAAATGTATAAATATGAATTATATTTACATG~~TTT~~
 3661 TTTAAGAAAAAAGTTACAAAAATATATACCAGGTACAGTAGTAAAGTGGCTGGTAG
 3721 TTGTCAATGGCTATAAAAAGAAATCTTATATTTTGCTATGTTTCAGTTTGTATTTT~~TT~~
 3781 AAATTATGTTCTAAGAC~~TTCTTAT~~CCCCAGTACAAGTCCCTA~~ATAAAG~~AGAGAATTGGC~~T~~
 3841 TAAGGTAAAAAAAAAAAAAAAAA

Figure 8. Protein Sequence Alignment of XFGFR-A3 with XFGFR-A1 and XFGFR-A2. The asterisk stands for conserved regions, dot for conservative changes and a space indicates non-conservative changes. Dashed line stands for amino acid deletion.

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XFGFR-A1  MFSGRSLLLGWVLLGAALSVARPPSTLPDEVAPKTKTEVEPY SARPGDTV
XFGFR-A3  MFSGRSLLLGWVLLGAALSVARPPSTLPDQVAPKTKTEVEPYSAQPGDRI
XFGFR-A2  MFSGMSLLLGWVLLGAALSVARPPSTLPDE-----
          ****.*****.

XFGFR-A1  TLQVRLREDVQSISWVKNGVQLLETNRTRITGEEIQISNAGPEDNGLYAC
XFGFR-A3  TLQCRRLREDVQSIWVKNGVQLSETNRTRITGEEIQISNAGPEDNGVYAC
XFGFR-A2  -----

XFGFR-A1  VTIGPSGTYTVLFSINVSDAQPSAEDDDDDDDNSSEEKASENSKPNRPF
XFGFR-A3  VTNGPSRTYTVLFSVNVSDALPSAEDDDDDDDNSSEEKAAENSKPNRPL
XFGFR-A2  -----DALPSAEDDDDDDDNSSEEKAAENSKPNRPL
          ** *****.*****.

XFGFR-A1  WSHPEKMEKKLHAVPAAKTVKFRCPANGTPSPALRWLKNKGEFRPDQRIG
XFGFR-A3  WSHPEKMEKKLHAVPAAKTVKFRCPANGTPTPTLRWLKNGRAFPQQDQRIG
XFGFR-A2  WSHPEKMEKKLHAVPAAKTVKFRCPANGTPTPTLRWLKNGRAFPQQDQRIG
          *****.*****.

XFGFR-A1  GYKVRSTWLSLIMDSVVP SDKGNVTCIVENKYGT LNHTYQLDVVERSPHR
XFGFR-A3  GYKVRSTWLSLIMDSVVP SDKGNVTCIVENKYGA INHTYQLDVVERSPHR
XFGFR-A2  GYKVRSTWLSLIMDSVVP SDKGNVTCIVENKYGA INHTYQLDVVERSPHR
          *****.*****.

XFGFR-A1  PILQAGLPANTSVTVGSTA EFSCKVYSDPQPHIQWLRHIEINGSRVASDG
XFGFR-A3  PILQAGLPANTSVTVGSTA EFSCKVYSDPQPHIQWLRHIEINGSRVASDG
XFGFR-A2  PILQAGLPANTSVTVGSTA EFSCKVYSDPQPHIQWLRHIEINGSRVASDG
          *****.*****.

XFGFR-A1  FPYVEILKTAGVNTSDK DMEVLHLRNVTFEDAGQYTCLAANSIGISHSHA
XFGFR-A3  FPYVEILKTAGVNTSDK DMEVLHLRNVTFEDAGQYTCLAANSIGISHSHA
XFGFR-A2  FPYVEILKTAGVNTSDK DMEVLHLRNVTFEDAGQYTCLAANSIGISHSHA
          *****.*****.

XFGFR-A1  WLTVLEVEDDKPALLAS PLQLEII IYCTGAAFVSAMVVTII IFKMKHPK
XFGFR-A3  WLTVLKVEDNKPALLAS PLQLEII IYCTGAAFVSAMVVTII IFKMKHPK
XFGFR-A2  WLTVLKVEDNKPALLAS PLQLEII IYCTGAAFVSAMVVTII IFKMKHPK
          *****.*****.

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XFGFR-A1 KSDFNSQLAVHKLAKSIPLRRQVTVSGDSSSSMHSGVILVRPSRLSSSGT
XFGFR-A3 KSDFNSQLAVHKLAKSIPLRRQV--SGDSSSSMNSGVILVR--RLSSSGT
XFGFR-A2 KSDFNSQLAVHKLAKSIPLRRQVTVSGDSSSSMNSGVILVR--RLSSSGT
*****

XFGFR-A1 PMLSGVSEYELPEDPRWEVARDRLILGKPLGEGCFQGVVMAEAIGLDKEK
XFGFR-A3 PMLSGVSEYELPEDPRWEVARDRLILGKPLGEGCFQGVVMAEAIGLDKEK
XFGFR-A2 PMLSGVSEYELPEDPRWEVARDRLILGKPLGEGCFQGVVMAEAIGLDKEK
*****

XFGFR-A2 PNKVTKVALKMLKSDANEKDLSDLISEMENNMIKHKHNIINLLGACTQD
XFGFR-A3 PNKVTKVALKMLKSDANEKDLSDLISEMENNMIKHKHNIINLLGACTQD
XFGFR-A2 PNKVTKVALKMLKSDANEKDLSDLISEMENNMIKHKHNIINLLGACTQD
**.....

XFGFR-A1 GPLYVIVEYASKGNLREYLRARRPPGMEYCYNPMCAFDQLLSFKDLVSCA
XFGFR-A3 GPLYVIVEYTSKGNLREYLRARRPPGMEYCYNPTCFDQLLSFKDLVSCA
XFGFR-A2 GPLYVIVEYTSKGNLREYLRARRPPGMEYCYNPTCFDQLLSFKDLVSCA
*****

XFGFR-A2 YQVARGMEYLASKKCIHRDLAARNVLVTDNVMKIADPGLARDIHIIHIDYY
XFGFR-A3 YQVARGMDYLASKKCIHRDLAARNVLVTDNIMKIADPALARDIHIIHIDYY
XFGFR-A2 YQVARGMDYLASKKCIHRDLAARNVLVTDNIMKIADPGLARDIHIIHIDYY
*****

XFGFR-A1 KKTNGRLPVKWMAPALFDRIYTHQSDVWSFGVLLWEIPTLGGSPYPGV
XFGFR-A3 KKTNGRLPVKWMAPALFDRIYTHQSDVWSFGVLLWEIPTLGGSPYPGV
XFGFR-A2 KKTNGRLPVKWMAPALFDRIYTHQSDVWSFGVLLWEIPTLGGSPYPGV
*****

XFGFR-A1 PHEELFKLLKEGHRMDKPTNCTNDVYMMKDCWHAMPSQRPTFNQLVEDL
XFGFR-A3 PHEELFKLLKEGHRMDKPTNCTNELYMMKDCWHAMPSQRPTFNQLVEDL
XFGFR-A2 PHEELFKLLKEGHRMDKPTNCTNDVYMMKDCWHAMPSQRPTFNQLVEDL
*****

XFGFR-A1 DRILALSSNQEYLDLSMPVNQYSPCFDTRSSSTCSSGEDSVFHDPLPDE
XFGFR-A3 DRILALSSNQEYLDLSMPVNQYSPCFDTRSSSTCSSGEDSVFHDPLPDE
XFGFR-A2 DRILALSSNQEYLDLSMPVNQYSPCFDTRSSSTCSSGEDSVFHDPLPDE
*****

XFGFR-A1 PCLPKYSNGGLKKR 814
XFGFR-A3 PCLPKYSNGGLKKR 810
XFGFR-A2 PCLPKYSNGGLKKR 724
*****

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XP_019A1 AAATTTCGAAATTAATTCGGAACAAATGAGTATGCTGTGTACTAACGG - 494
 XP_019A2 -----ATGCACCTGCCT - 255
 XP_019A1 - GCTTCTCGAACATATACAGTATTATCTCCGTTAATGTATCAGATGCACCTGCCT - 549
 XP_019A2 - TGTGCCGAGACGATGATGAATGATGACAAATTCATCTCTGAGGAGAAAGCTG - 310
 XP_019A1 TGTGCCGAGACGATGATGAAGATGATGACAAATTCATCTCTGAGGAGAAAGCTG - 604
 XP_019A2 - CAGAAACTTCAAAACGAAACGCTCCACTTTGGTCACATCCAGAAAAATGGAGAA - 365
 XP_019A1 - CAGAAACTTCAAAACGAAACGCTCCACTTTGGTCACATCCAGAAAAATGGAGAA - 659
 XP_019A2 GAAATTCATGCACTGCCAGCAAAAACTGTAAATTTAGGTGCCAGCAAT - 420
 XP_019A1 GAAATTCATGCACTGCCAGCAAAAACTGTAAATTTAGGTGCCAGCAAT - 714
 XP_019A2 GTAACTTAACGCAACACTTCGCTGCGTGAAGAATGGCAGGCGTTCCAACAAG - 475
 XP_019A1 - GTAACTCCAACGCCAACACTTCGCTGCGTGAAGAATGGCAGGCGTTCCAACAAG - 769
 XP_019A2 - ATTAATGATTTATTAATTAAGGTCCTCAACATGAGTCTTATTATGGA - 530
 XP_019A1 ATTAAGGCAATTTGCTTAATTAAGGTCCTCAACATGAGTCTTATTATGGA - 824
 XP_019A2 TTTCTGTGTCCTATCTGATAAAGGAAATTTACACCTGTATTGTGGAGAACAGTAT - 585
 XP_019A1 - TTTCTGTGTCCTATCTGATAAAGGAAATTTACACCTGTATTGTGGAGAACAGTAT - 879
 XP_019A2 - GTGATATCAACACACATACCACTTAGATTTGTTGAGCGTCCCCACACCGCC - 640
 XP_019A1 - GTGATATCAACACACATACCACTTAGATTTGTTGAGCGTCCCCACACCGCC - 934
 XP_019A2 - CAATTTTACAAAGCGCTCTCCAGCGAATACAAAGCTTACTGTGGAAACACAGC - 695
 XP_019A1 - CAATTTTACAAAGCGCTCTCCAGCGAATACAAAGCTTACTGTGGAAACACAGC - 989
 XP_019A2 - CCAATTTTCTGCAAGGTGTACAGCGACCCCGAGCTCACATCCAATGGCTCAGG - 750
 XP_019A1 - CCAATTTTCTGCAAGGTGTACAGCGACCCCGAGCTCACATCCAATGGCTCAGG - 1044

XPGFRA2	- CACATTGAAATTAAAGACACAAAGTGGACTTGGATGGATTTCCTGATATGAGAGAA	806
XPGFRA3	- CACATTGAAATTAAAGACACAAAGTGGACTTGGATGGATTTCCTGATATGAGAGAA	1099
XPGFRA2	- TCCTCAAGACTGCAGAGCTCAACACTCTGAAAGGATATGAGAGTTTTCATTAAT	860
XPGFRA3	- TCCTCAAGACTGCAGAGCTCAACACTCTGAAAGGATATGAGAGTTTTCATTAAT	1154
XPGFRA2	- GAGAAATGTTACTTTTGAGATGCTGGCTCAATATACCTGTTGAGGATTAATTC	915
XPGFRA3	- GAGAAATGTTACTTTTGAGATGCTGGCTCAATATACCTGTTGAGGATTAATTC	1209
XPGFRA2	- ATTGGGATCTCTCATCATCTCTGCATAGTTGACCTTTTAAAGTTGAGAGGAATA	970
XPGFRA3	- ATTGGGATCTCTCATCATCTCTGCATAGTTGACCTTTTAAAGTTGAGAGGAATA	1264
XPGFRA2	- AACCTGGGCTTCTGGGCTCCCTTTTACAACCTGAAATTATATATATGAGAGAG	1025
XPGFRA3	- AACCTGGGCTTCTGGGCTCCCTTTTACAACCTGAAATTATATATATGAGAGAG	1419
XPGFRA2	- GGCTGCTTTTGTTGGCGAATGTTGTTGACATATTATTTTAAATGAGAGAA	1080
XPGFRA3	- GGCTGCTTTTGTTGGCTGGAATGTTGTTGATCATTATTTTAAATGAGAGAA	1474
XPGFRA2	- CCGTCGAGGAAGTCGACTTCAACAGCTTAACTGTTGTTGAGAGAGTTGTTGAGAA	1135
XPGFRA3	- CCGTCGAGGAAGTCGACTTCAACAGCTTAACTGTTGTTGAGAGAGTTGTTGAGAA	1429
XPGFRA2	- GCATCCCGCTCGCGACACAGCTTAACTGTTGTTGAGAGAGTTGTTGAGAA	1190
XPGFRA3	- GCATCCCGCTCGCGACACAGCTTAACTGTTGTTGAGAGAGTTGTTGAGAA	1476
XPGFRA2	- CTCCTGGAGTGATATTAGTCAACGCTTTCTTCAGTTGTTGAGAGTTGTTGAGAA	1245
XPGFRA3	- CTCCTGGAGTGATATTAGTCAACGCTTTCTTCAGTTGTTGAGAGTTGTTGAGAA	1544
XPGFRA2	- GGACTATCGGAATATGAGCTTCCAGAGATCCAGATGTTGAGAGAGTTGTTGAGAA	1300
XPGFRA3	- GGACTATCGGAATATGAGCTTCCAGAGATCCAGATGTTGAGAGAGTTGTTGAGAA	1598
XPGFRA2	- GACTGATCCTTGGGAAACCTCTCGAGAGTTGTTGAGAGAGTTGTTGAGAA	1355
XPGFRA3	- GACTGATCCTTGGGAAACCTCTCGAGAGTTGTTGAGAGAGTTGTTGAGAA	1644

XP_009A2 GAGGGTATTGTTTCTGACAAATGAGAAAGCTAACAAATTAACAAAATTGCTGTG - 1410
 |||
 XP_009A3 - GAGGGTATTGTTCTGACAAATGAGAAAGCTAACAAATTAACAAAAGTTGCTGTG - 1698
 |||
 XP_009A2 AATATGTTTAAATCTATATGGAATGAAAAATACCTGTGGATCTGATTTCCGAGA - 1465
 |||
 XP_009A3 AATATGTTTAAATCTGATGCGAGTAAAGAGACCTGTGGATCTGATTTCCGAGA - 1753
 |||
 XP_009A2 TTTAAATGATGAAATGATTGAAAAACAAAAATATAATTAAATTTACTTGGTGC - 1520
 |||
 XP_009A3 TTTAAATGATGAAATGATTGAAAAACAAAAATATAATTAAATTTACTTGGTGC - 1808
 |||
 XP_009A2 CTGACCTAAATGTTTCACTTATGTAATTGTAATACACTTCCAAAGGGAAT - 1575
 |||
 XP_009A3 - CTGACCTAAATGTTTCACTTATGTAATTGTAATACACTTCCAAAGGGAAT - 1863
 |||
 XP_009A2 CTGAAATGATTTTAAAGGCGCGCGCGCGGATGGAGTACTACTACAACC - 1630
 |||
 XP_009A3 CTGAAATGATTTTAAAGGCGCGCGCGCGGATGGAGTACTACTACAACC - 1918
 |||
 XP_009A2 - CTAACTTGTGCGGATTAAGCTGTTTCTCTCAAGATCTGGTGTGATGTGCTTA - 1685
 |||
 XP_009A3 CTAACTTGTGCGGATTAAGCTGTTTCTCTCAAGATCTGGTGTGATGTGCTTA - 1973
 |||
 XP_009A2 CTAAGTTGCAATGTTGATGACTAGCTCTAAAAATGATCCACAGAGAC - 1740
 |||
 XP_009A3 - CTAAGTTGCAATGTTGATGACTAGCTCTAAAAATGATCCACAGAGAC - 2028
 |||
 XP_009A2 CTGACTGAAGGAATTTTGTAAACAGAGGACAACATAATGAAGATTGCCGATT - 1795
 |||
 XP_009A3 - CTGACTGAAGGAATTTTGTAAACAGAGGACAACATAATGAAGATTGCCGATT - 2083
 |||
 XP_009A2 TTTGTTAAGCTTGACATTCATCATTGACTATTATAAGAAAACGACAAATGG - 1850
 |||
 XP_009A3 TTTGTTAAGCTTGACATTCATCATTGACTATTATAAGAAAACGACAAATGG - 2138
 |||
 XP_009A2 CTTGCTGCTGTAATAATGGATGATCCAGAGGACCTGTTGACCGGATTACACT - 1905
 |||
 XP_009A3 CTTGCTGCTGTAATAATGGATGATCCAGAGGACCTGTTGACCGGATTACACT - 2193
 |||

XFGFRA2	- CATCAGAGCGATGTGTAGTCATTTACGCTACTATCTNAGAGATTTTAACTTA	1960
XFGFRA3	- CATCAGAGCGATGTGTAGTCATTTACGCTACTATCTNAGAGATTTTAACTTA	2248
XFGFRA2	- GTGGCTCCCATATCCGAGTGTCCCATGGAAGAACTCTTTAAATTACTTAAGAA	2015
XFGFRA3	- GTGGCTCCCATATCCGAGTGTCCCATGGAAGAACTCTTTAAATTACTTAAGAA	2303
XFGFRA2	- AGGCGACAGGATGATATAACCCACTAACTGACCAATGATTTATATATGATAT	2070
XFGFRA3	- AGGCGACAGGATGATATAACCCACTAACTGACCAATGATTTATATATGATAT	2368
XFGFRA2	- AAGGACTGTTGGCATGCCATGCGCTCTTAAAGAACAACTTAATTAATTAATTA	2125
XFGFRA3	- AAGGACTGTTGGCATGCCATGCGCTCTTAAAGAACAACTTAATTAATTAATTA	2413
XFGFRA2	- AGGATCTTGACCGAATCTCTACTGATTTCCAAATAGGAATATCTGATCTCT	2180
XFGFRA3	- AGGATCTTGACCGAATCTCTACTGATTTCCAAATAGGAATATCTGATCTCT	2468
XFGFRA2	- CATGCCAGTGAATCAATATCTCATGTTTCTTAAAGATTAAGATTAAGATTA	2235
XFGFRA3	- CATGCCAGTGAATCAATATCTCATGTTTCTTAAAGATTAAGATTAAGATTA	2523
XFGFRA2	- TCTTCAGGCGAGGACTCTGTCTTCTCATTAAGCTTAAAGATTAAGATTAAG	2290
XFGFRA3	- TCTTCAGGCGAGGACTCTGTCTTCTCATTAAGCTTAAAGATTAAGATTAAG	2578
XFGFRA2	- TTCCCAATACTCCAAATGGTGGACTTAAAGAAAGCTTAAGCTTAAGCTTAAG	2345
XFGFRA3	- TTCCCAATACTCCAAATGGTGGACTTAAAGAAAGCTTAAGCTTAAGCTTAAG	2633
XFGFRA2	- GGTTCTTCTCCATAAATTCATTTAAAGAACTTTTAAAGATTAAGATTAAG	2399
XFGFRA3	- GGTTCTTCTCCATAAATTCATTTAAAGAACTTTTAAAGATTAAGATTAAG	2688
XFGFRA2	- ATGCCACAAGTTCCTCAGAAAGCATGTACAAATTCACCTCTACATCTCAATCT	2454
XFGFRA3	- ATGCCACAAGTTCCTCAGAAAGCATGTACAAATTCACCTCTACATCTCAATCT	2742
XFGFRA2	- ATTCTTTTACACATGAGTTTGTAGAGATATTTTCTTAAAGATTAAGATTAAG	2509
XFGFRA3	- ATTCTTTTACACATGAGTTTGTAGAGATATTTTCTTAAAGATTAAGATTAAG	2798

XP_03FA.1 2564
 ZP_03FA.1 2563
 XP_03FA.2 2619
 ZP_03FA.2 2608
 XP_03FA.2 2674
 ZP_03FA.1 2663
 XP_03FA.2 2729
 ZP_03FA.1 3016
 XP_03FA.2 2753
 ZP_03FA.1 3073
 XP_03FA.1 3128
 XP_03FA.1 3183
 XP_03FA.1 3238
 XP_03FA.1 3293
 XP_03FA.3 3348
 XP_03FA.1 3403
 XP_03FA.3 3458
 XP_03FA.1 3513
 XP_03FA.3 3568

XPGFRA3 - ATAAAAATGGAGTGGGAGAGGAATGGAATAATGAGTTGCTTTGATATAAGTAA 9678
 XPGFRA3 - AAAATGTATAAATATGAATTATATATTTAATGKCTTTTAAAGAAAAA 9678
 XPGFRA3 - GTTACAAAATATATATAGAGGAGTAAGAAATGAGTGTAGAGGAGGAATGTA 9700
 XPGFRA3 - TAAAAAGAAATTTATATTTTGTATCTTTTAAATTTATTTTTTAAATTATG 9788
 XPGFRA3 - TTCTAAAGCTTTTATTGAGAGTAAGAGTGTGTAAGTAAAGGAATGAGTTAA 9840
 XPGFRA3 - GGTAAAAA 9840

Identity : 2734 (99.3%)

Figure 10. RT-PCR analysis of XFGFR-A3 expression with DD-1 and S402 oligonucleotide primers.

Figure 10A. Schematic diagram showing RT-PCR technique.

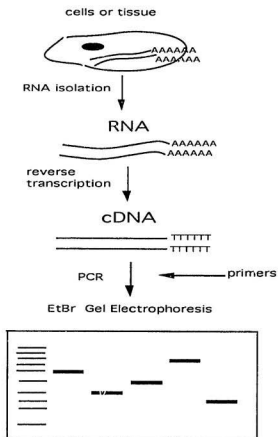


Figure 10B. Schematic diagram showing the locations of PCR primer T305, DD-1, Px-2, and S402 on XFGFR-1. Probe used for screening the library is also shown. The restriction enzyme sites, and Val⁴²³-Thr⁴²⁴ dipeptide location are indicated by arrows.

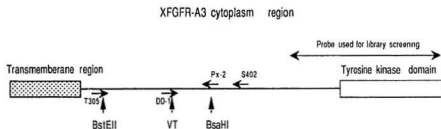


Figure 10C. cDNAs from six different stage *Xenopus* embryos, along with XFGFR-A3 plasmid, were PCR amplified with DD-1 and S402 primers (to recognize the XFGFR-A3 sequence only) or with T305 and S402 primers (to recognize all XFGFR-1/*flg* variants). T305 and S402 primers are used as a PCR amplification control. PCR was performed as described in the Materials and Methods section. Total RNA was isolated from different stage *Xenopus* embryos and cDNA was synthesized using an oligo (dT) primer. Following 32 cycles of PCR amplification, 10 % of each PCR product was run on a 2 % agarose gel in 1xTBE buffer. The results indicated that XFGFR-A3 was expressed in all stages of *Xenopus* embryos. Lane 1, 3, 5, 7, 9 and 11 are RT-PCR products from stage 2, 6, 8, 24, 30 and 41 embryos with T305 and S402 as primers. Lane 2, 4, 6, 8, 10 and 12 are PCR products from stage 2, 6, 8, 24, 30 and 41 embryos with DD-1 and S402 as primers. Lane 13 is RT-PCR product from XFGFR-A3 plasmid with DD-1 and S402 as primers. Lane 14 is RT-PCR product from XFGFR-A3 plasmid with T305 and S402 as primers. Lane 15 is 1 Kb ladder DNA size markers.

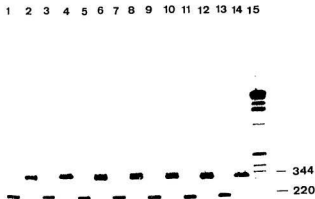


Table 5. Oligonucleotide primers used in the sequencing and RT-PCR experiments.

Name	sequences (5' to 3')	Location in XFGFR-A3
T3-4	ACA AGC CGG TCT CCC AGC GAA T	953-974
T7-4	GTC TTA GAT GGT AGC CTG GAG T	3016-3037
DD-1	GCG CAG ACA GGT TTC AGG GG	1395-1457
T3-3	GLT TCA TGC AGT GCC AGC	663-680
S3-3	CCT AAT GTT GAC CTC CAG	2808-2825
T3-5	GGG CTG CTT TTG TGT CCG CAA T	1319-1340
S3-5	GAC TGA AG(G)C GTG TGT TTC AGT G	3444-3465
S3-4	CTC CTT GTA GCT AGG TTA GAC T	3118-3139
S4-4	GTT GAC TCC TGC AGT CTT GAG G	1101-1122
T7-3	GGT TGC ATA ATG AGC ACA	3335-3352
S4-3	TTG TGC ACA GCC AGT TGG	1401-1418
S3-2	CTC GAA GTT CGA CGT G	2507-2522
S4-2	GCC ATG ACT ACT TGC C	1628-1643
S4-5	TGC CAT TCT TCA GCC AGC GAA G	733-754
T7-5	TGC TTC TGA GGA ACT TGT GGC A	2690-2710
PX-1	CGC TGC GCA GAC AGG TAA CAG T	N/A
PX-2	CAC TGG AAG AAA GGC GCG AAG G	1494-1513
T3-6	GCT ATT GGC CTG GAC AAG GAG A	1626-1648
T7-6	ATG GCA TGC CAA CAG TCC TTC A	2357-2379
T3	GCT CGA AAT TAA CCC TCA CTA AAG	N/A
T7	GGT ACC TAA TAC GAC TCA CTA TAG GG	N/A
T3-2	AGA GCA TCA ACT GGT G	365-380
T7-2	CTA CTG ACC TGG TAT A	3690-3705
PO-1	GAG AAT TCG TCG ACA TCG ATT TTT TTT	N/A
	TTT TTT TT	

polyadenylation element and the hexanucleotide polyadenylation signal (AAUAAA).

A unique feature of the XFGFR-A3 clone is the Val⁴²³-Thr⁴²⁴ dipeptide deletion caused by 6 nt deletion located in the juxtamembrane region of XFGFR-A3, which could result in the loss of a putative serine/threonine phosphorylation site. A similar variant of the human *flg* gene has been identified (Hou et al., 1991). This is the first time such a variant has been described in *Xenopus*. XFGFR-A3 shows more homology at the amino acid level to XFGFR-A2 than to XFGFR-A1 (Fig 8). XFGFR-A3 and XFGFR-A2 have identical 5' non-coding sequence which indicates they are likely the same gene products (Figure 9). Therefore XFGFR-A3 is a variant of XFGFR-A2. The main differences between XFGFR-A2 and XFGFR-A3 are: 1) XFGFR-A3 is a three Ig-like domain form and XFGFR-A2 is a two Ig-like domain form. 2) XFGFR-A3 has a 6 nt deletion at juxtamembrane region which can cause Val⁴²³-Thr⁴²⁴ dipeptide deletion. In addition, there are five single amino acid differences between A2 and A3. The XFGFR-A3 nucleotide sequence and deduced amino acid sequence are described in Figure 7.

3.3. RT-PCR Analysis of XFGFR-A3 and XFGFR-A2 Expression during *Xenopus* Embryonic Development

The DD-1 primer (Figure 10B, Table 5) was designed according to Val⁴²³-Thr⁴²⁴ dipeptide deleted region of XFGFR A3. Px-1 was designed according to the same region in XFGFR A2 (Figure 10B). Px-2, T305 and S402 primers are located outside this region and recognize both XFGFR-A3 and XFGFR-A2. DD-1 and S402 primers were used in the PCR amplification to specifically amplify FGFR-A3 cDNA while Px-1 and Px-2 primers specifically amplify the same region on FGFR-A2. T305 and S402 primers were used in each experiment to ensure that the RT-cDNA used as a template contains the region of interest; they amplify fragments of 326 bp and 330 bp in size from XFGFR-A2 and XFGFR-A3, respectively, and cover the region that the primer pairs DD-1 and S402 or Px-1 and Px-2 amplify.

The RT-PCR results shown on Fig.10C and 13 demonstrated that both XFGFR-A3 and XFGFR-A2 mRNA are present at all stages of *Xenopus* development. Although RT-PCR is a very sensitive method and has many advantages over Northern analysis in terms of detecting gene expression, it is very difficult to

quantitate, even with an internal PCR control (Siebert and Larrick, 1992). Therefore, to obtain information about the ratio of each XFGFR1 isoform in the whole XFGFR1 mRNA population and a detailed expression pattern of each, RNase protection assays were performed.

3.4. Probe Construction for RNase Protection

The RNase protection probe was obtained by subcloning a 162 bp BstEII-BsaHI cDNA fragment covering the VT dipeptide deletion region of XFGFR-A2 into the EcoRV site of pBluescript KS(+)(Stratagene, La Jolla, CA , USA) by blunt-end ligation. The location of this 162 bp XFGFR-A2 fragment in XFGFR-A2 is shown in Figure 11; it covers nucleotides 1046 to 1212 of XFGFR-A2. Restriction enzymes used in this construction and other subcloning manipulations are listed in Table 6.

Transformation of competent E.coli XL-Blue cell was performed as described in Methods (2.12.). The orientation of the insert was checked by PCR with T7 and S4-3 primer pairs. Transformants that gave full-length inserts were subjected to large scale plasmid preparation, as described in methods (2.12.). The purified 162 bp XFGFR-A2 plasmids were verified by sequencing.

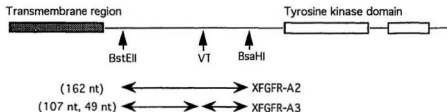
Table 6. Restriction endonucleases used and their recognition sites.

BamHI	G/GATCC
BsaHI	GR/CGYC
BstEII	G/GTNACC
EcoRI	G/AATTC
EcoRV	GAT/ATC
HindIII	A/AGCTT
SpeI	A/CTAGT
NotI	GC/GGCCGC
XhoI	C/TCGAG

"/" indicates the point of cleavage within a recognition sequence.

R= A or G N=A or C or G or T

Figure 11. Diagrammatic representation of probes used in the RNase protection assays. The locations for the RNase protection probe with respect to the juxtamembrane region and the expected protection fragments for both the XFGFR1-A3 and XFGFR-A2 transcripts are shown. Arrows indicate the locations of the restriction enzyme sites and the location of Val⁴²³-Thr⁴²⁴ dipeptide in the juxtamembrane region. The probe is a 162-nt BstEII/BsaHI digested XFGFR1-A2 cDNA subclone.



3.5. RNase Protection Assay to Examine XFGFR-A3 Expression During *Xenopus* Embryonic Development

The probe used for the RNase protection experiments was prepared by subcloning the corresponding XFGFR-A2 plasmid region into KS(+) plasmid as mentioned above (3.5.). To generate a XFGFR-A2 probe for RNase protection, the plasmid was linearized by XhoI (located in the polylinker region). A [α - 32 P]UTP labelled antisense probe was generated by *in vitro* transcription from the T7 promoter. This probe is 246 bases and protects a 162 bp fragment in FGFR-A2 and two fragments of 107 bp and 49 bp in FGFR-A3 (Figure 11).

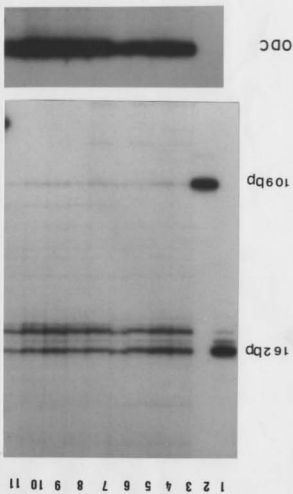
To serve as a positive control for the protection assay, plasmids containing the full length XFGFR-A3 and XFGFR-A2 cDNAs were linearized at KpnI site and XhoI site, respectively, and subjected to *in vitro* transcription with T3 RNA polymerase. RNase protection assays of XFGFR-A2 and XFGFR-A3 cRNA enabled me to verify that the protected fragments were of predicted size and that the digestion was complete.

A KS(+) plasmid containing 420 bp insert of ODC was digested with XhoI and transcribed by T7 RNA polymerase to

produce an antisense RNA probe [The 420 bp *Xenopus* ODC (Ornithine decarboxylase) plasmid is a gift from Dr. J. M. Slack to Dr. G. D. Paterno.]. This probe is 420 bases and can protect ODC transcripts to yield a fragment of 327 bp. This ODC probe served as a RNA loading control probe to detect the uniformly expressed ODC-1 transcripts in all different stages of *Xenopus* embryos.

Using RNase protection assays, I analyzed XFGFR-A3 and XFGFR-A2 expression quantitatively throughout *Xenopus* development. Consistent with the RT-PCR results, both XFGFR-A3 and XFGFR-A2 mRNA were present throughout *Xenopus* development. The level of expression for each form of the XFGFR was constant through the stages examined. The results also showed that XFGFR-A3 was expressed at a much lower level than XFGFR-A2(Figure 12). There were two protected bands for XFGFR-A2. It could be caused by the secondary structure formed between XFGFR-A2 mRNA and antisense probe at the 3' end of the duplex; Because this protection pattern was also present in the XFGFR-A2 mRNA control samples. By scanning and comparing the 107 and 162 bp bands on autograph (GelScan XL), I determined that the mRNA copy number of XFGFR-A3 was 5.5 % of the XFGFR-A2.

Figure 12. RNase protection assay of XFGFR-A3 expression during *Xenopus* embryonic development. The assay was performed as described in Materials and Methods. 10 μ g total RNA from different stage embryos were hybridized with [γ - 32 P] dATP labelled XFGFR-A2 probes (5×10^5 cpm). The ODC-1 probe (5×10^3 cpm) was included with each RNase protection sample to monitor RNA loading equivalency. The autoradiograph was exposed to Kodak XAR-5 film for 48 hours with intensifying screen. Arrows indicate the fragment size of corresponding DNA band. Lane 1: *in vitro* transcribed cRNA strand from XFGFR-A2 protected by the XFGFR-A2 probe. Lane 2: *in vitro* transcribed cRNA strand from XFGFR-A3 RNA protected by the XFGFR-A2 probe. Lane 3, 4, 5, 6, 7, 8, 9, 10, and 11 are total RNAs from stage 2, 6, 8, 10, 16, 24, 30, 36, and 42 embryos respectively and protected by the XFGFR-A2 and ODC probe.



3.6. Genomic DNA Approach to Analyze the Nucleotide Sequences of the VT Dipeptide Deletion Region of XFGFR-A3

To investigate the cause of the VT dipeptide deletion in XFGFR-A3, sequence analysis of genomic DNA was performed. PCR of genomic DNA is a fast and efficient way to obtain sequence information of the genomic DNA flanking the VT deleted region. A *Xenopus* genomic library (Stratagene, La Jolla. USA) was used as a substrate in the PCR amplification. The principle of this genomic PCR method is shown in Figure 14A. Two primers T305 and S402 (Table 5) were used to amplify the XFGFR1 genomic DNA corresponding to the region. A 1.2 Kb band is amplified from the genomic DNA while a 300 bp band was amplified from the cDNA library. The amplified XFGFR1 genomic DNA fragment was gel-purified and subjected to PCR sequencing. Comparison of the genomic DNA sequence, the cDNA sequence and the amino acid sequence, revealed that this VT dipeptide deletion site is located at an exon/intron boundary (Figure 14,15). By comparison with consensus sequences for 5' splice donor and 3' splice acceptor, I predict that alternative splicing caused this VT deletion in XFGFR-A3.

Figure 13. RT-PCR assay of XFGFR-A2 expression using Px-1 and Px-2 oligonucleotide primers. cDNAs from six different stage *Xenopus* embryos, along with the XFGFR-A2 plasmid, were PCR amplified with Px-1 and Px-2 primers (recognize the XFGFR-A2 sequence only) or with T305 and S402 primers (to recognize all the XFGFR-1/flg variants). T305 and S402 primers are used as a PCR amplification control. PCR were performed as described in Materials and Methods. Total RNA was isolated from different stage *Xenopus* embryos and cDNA was synthesized using oligo (dT) priming method. Following 32 cycles of PCR amplification, 10 % of each PCR product was run on a 2 % agarose gel in 1xTBE buffer. The results indicated XFGFR-A2 was also expressed in all stages of *Xenopus* embryos. Lane 1, 3, 5, 7, 9 and 11 are RT-PCR products from stage 2, 6, 8, 24, 30 and 41 embryos with T305 and S402 as primers. Lane 2, 4, 6, 8, 10 and 12 are PCR products from stage 2, 6, 8, 24, 30 and 41 embryos with Px-1 and Px-2 as primers. Lane 13 is RT-PCR product from XFGFR-A3 plasmid with T305 and S402 as primers. Lane 14 is RT-PCR product from XFGFR-A3 plasmid with Px-1 and Px-2 as primers. Lane 15 is 1 Kb ladder DNA size markers.

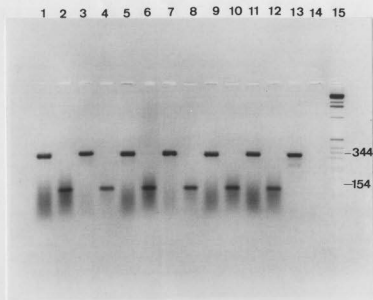


Figure 14. Genomic DNA PCR approach to characterize Val⁴²⁴-Thr⁴²⁴ deletion region of XFGFR-A3.

Figure 14A. Schematic diagram of the genomic PCR method. The PCR primers are constructed based on exon sequences. If the genomic sequence contains one or more introns between the primer sequences, the PCR products from the cDNA template will be smaller than the PCR products derived from the genomic DNA.

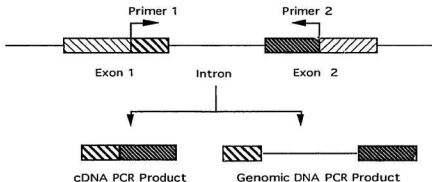


Figure 14B. Comparison of PCR-amplified XFGFR-1/*flg* fragment from *Xenopus* cDNA Libraries (lane 2 and 3) and genomic DNA libraries (Lane 1). Lane 4 contains 1 kb ladder DNA size markers. PCR primers are T305 and S402 which span a intron in FGFR1/*flg* genomic DNA. The PCR amplified genomic DNA fragment is 1.4 kb while the cDNA fragment is 330 bp.

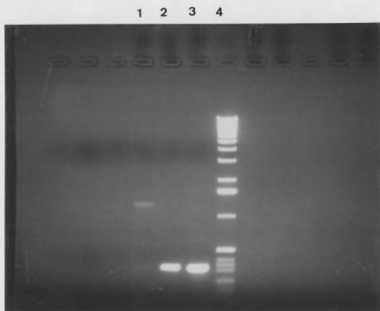


Figure 15. XFGFR1-A3 genomic DNA sequences spanning the Val⁴²³-Thr⁴²⁴ deletion region. The nucleotide sequences of XFGFR-A3 genomic fragment containing Val⁴²³-Thr⁴²⁴ deletion are shown in bold. Exon sequences are shown in uppercase and intron sequences are shown in lowercase. Corresponding amino acid sequence is also shown. The nucleotides with asterisk indicate the alternative 5' splice donor sites involved in the alternative splicing process to generate the Val⁴²³-Thr⁴²⁴ deletion.

```

      *      *
CTG CGC AGA CAGgtatcagaagatatttaggggttttcc.....cggcgggtcttcttctctgggaatggtttt TGA tggat gaa*
L  R  R  Q  V  T                               V  S  G  D

```


Figure 16. Stained protein gel showing expression of GEX-FGFR(VT+), GEX-FGFR(VT-) fusion protein and GST. Lane 1 is molecular-weight marker for size indication. Lane 2 and 3 are GST protein after elution from glutathione-agarose beads. lane 4 and 5 are GEX-FGFR(VT+) fusion protein after elution from glutathione-agarose beads. Lane 6 and 7 are GEX-FGFR(VT-) fusion protein after elution from glutathione-agarose beads. Lane 9 and 10 are the total cell lysate of pGEX-KT transformant after IPTG induction. Lane 11 and 12 are the total cell lysate of pGEX-FGFR(VT+) transformant after IPTG induction. Lane 13 and 14 are the total cell lysate of pGEX-FGFR(VT-) transformant after IPTG induction. Lane 15 is molecular-weight marker for size indication.

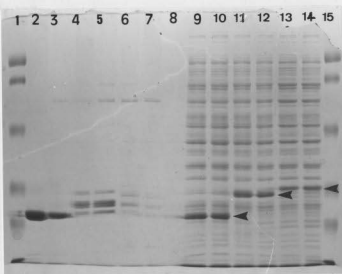


Table 7. Results from PKC assay.

	Total ³² p incorporation (CPM/nmol protein)	PKC-specific incorporation (CPM/nmol protein)
GEX-FGFR(VT+)	12,885	6,457
GEX-FGFR(VT+)+ PKC inhibitor	6,428	
GEX-FGFR(VT-)	7,011	0
GEX-FGFR(VT-)+ PKC inhibitor	8,161	

3.7. PKC Assay Results of GST-FGFR(VT+) and GST-FGFR(VT-) fusion proteins

The GST-FGFR(VT-) and GST-FGFR(VT+) fusion proteins were purified as outlined in Methods (2.2.13.2). The purity of the fusion protein was analyzed on SDS-PAGE (Figure 16). There were contaminants. But for preliminary *in vitro* phosphorylation assay, I only wished to see if there was a difference between the two fusion protein preparations. Thr⁴²⁴ of XFGFR1 is contained within a consensus phosphorylation site for PKC (Kennelly and Krebs 1991). PKC requires basic amino acid residues near the phosphoacceptor group. PKC can be influenced by both N- and C- terminal basic residues. The sites of phosphorylation contains at least one arginine or lysine at position -1 through -3. Some possessed one or more at position +1 through +3. Arginine has been observed superior to lysine. The consensus sequences for PKC is given as $R/(K_{1-3}, X_{2-6}) S^*/T^*(X_{2-6}, R/K_{1-3})$. Where two amino acids function interchangeably, both are listed with a slash " / " separating them. Sequence position judged to be recognition neutral are denoted by an X. The phosphoacceptor amino acid is denoted by an asterisk. To test the possibility that Thr⁴²⁴ can be phosphorylated by PKC, I made two constructs using a portion of the juxtamembrane region of FGFR (detailed in

Methods 2.2.13). Construct pGEX-FGFR(VT-) expressed the fusion protein GST-FGFR(VT-) with Val⁴²³-Thr⁴²⁴ dipeptide deletion in the FGFR region while fusion protein expressed by the other construct pGEX-FGFR(VT+) had Val⁴²³-Thr⁴²⁴ in FGFR region. Protein Kinase C Assay System (GIBCO-BRL) and purified PKC (GIBCO-BRL) were used to measure the phosphorylation level of both GST fusion protein. The primary PKC assay result is shown in Table 7. PKC-specific ³²P incorporation in GST-FGFR(VT+) was ,457 cpm/nmol protein. Almost no ³²P incorporation was observed in GST-FGFR(VT-) with the presence of two extra threonine residues (Table 7). There were little amount of contaminating proteins in the preparation. So that at this point, I cannot eliminate the possibility that the observed phosphorylation was of a protein other than the fusion protein. Future experiments demand better purification of the fusion proteins. These preliminary data indicate that Thr⁴²⁴ is a potential PKC phosphorylation site.

Chapter 4

Discussion

4.1. Regulation of Mesoderm Formation by the FGF/FGFR Signalling System

Available evidence suggests that FGF plays an important role during mesoderm induction in *Xenopus* embryonic development (Slack et al, 1987; Gillespie et al, 1989; Amaya et al, 1991; Ryan and Gillespie, Submitted). It has been shown that not only can FGF induce mesoderm formation but also different concentrations of FGF induce different mesoderm tissues (Smith and Slack 1983; Slack. et al., 1984 ; Slack et al, 1987; Gillespie et al, 1989; Kimelman. et al., 1992). Therefore, it is very important to understand how the different responses can be mediated by FGF/FGFR signaling system during the mesoderm induction process.

There are several possible mechanisms which FGF/FGFR signalling system might use to generate different intracellular signals to induce differentiation into distinct

mesodermal cell types: 1) The activation of FGFRs might depend on the availability of the appropriate FGF family members (Giordano et al., 1992). Since all members of the FGF family bind to the extracellular matrix, their distribution is likely to be restricted to the area surrounding the cells that produce them (Klagsbrun et al., 1986). The distribution of the FGFs during embryonic development have been studied in several species (Goldfarb, 1990). RNAs for aFGF and bFGF have been detected in mouse embryo throughout gestation. RNAs for aFGF and bFGF have also been found in blastula stage amphibian embryos. The most thoroughly characterized expression profile for FGF genes is that of murine *int-2*. *int-2* RNA is expressed throughout embryogenesis and at birth but not in any adult tissues. The murine FGF5 and FGF6 genes are expressed throughout embryogenesis as well as in restricted sets of adult tissues. It is possible that the activation of FGF receptors in embryos can be regulated by the availability of the appropriate FGF ligand (Giordano et al., 1992). 2) FGF/FGFR signalling system can generate different intracellular signals by regulating the availability of various members of the FGFR family in different cells or embryonic tissues (Patstone et al., 1993). The distribution of FGF receptors during development had been characterized in human and other

species by *in situ* hybridization. FGFR1 was found highly expressed in the skin, brain, and bone of chickens, mice, and humans embryos (Wanaka *et al.*, 1991; Safran *et al.*, 1990; Partanen *et al.*, 1991). The human FGFR 2 genes were expressed at high levels in skin and other epithelial cells (Peters *et al.*, 1992). FGFR3, which is closely related to FGFR2, was localized to human fetal brain, skin, bones, intestine, and lung (Partanen *et al.*, 1991). FGFR4 was shown to be expressed at high level in the adrenal and lung (Partanen *et al.*, 1991). Gillespie *et al.*, (1989) provided evidence that all cells of the *Xenopus* blastula have high affinity binding sites for radioiodinated FGF. In addition, the FGF/FGFR complex was phosphorylated on tyrosine residues in FGF treated *Xenopus* blastula cells (Gillespie *et al.*, 1992). As in other receptor tyrosine kinases, phosphorylation on tyrosine residues may activates the FGFR. It means that each of the FGFRs has a different biological role and highlights the importance of the spatial and temporal distribution of FGFRs in tissues and amphibian embryos in mediating different cellular responses. 3) The FGF receptors might mediate different cellular responses depending on the composition of the FGF receptor oligomer formed. Both FGFR1 and FGFR2 have the alternatively spliced isoforms with two Ig-like domain in the extracellular region

of the receptor. These isoforms have different ligand binding specificities (Werner *et al.*, 1992; Dionne *et al.*, 1990). In addition, the subtle differences in FGF receptor cytoplasmic domains might be responsible for the generation of different intracellular signals and cause different cellular responses. 4) The presence of other molecules, such as *Wnt* and *Noggin* family members, can also synergize with FGFs and change the cell responses to FGF/ FGFR signalling system (Christian *et al.*, 1992). It is also important to emphasize that probably each one of the four possibilities mentioned above has a role to play. By combining one or more of the mechanisms, the cellular response may be "fine tuned". The end result would be an "infinite" variety of cellular responses to FGF.

4.2. Expression of the FGFR1 Isoforms during *Xenopus* Development

Our interest was to investigate the relationship between the expression of different FGFRs and mesoderm induction in *Xenopus*. Up to now, cDNA clones of the FGFR1 (XFGFR-A1 and XFGFR-A2) were isolated from the XTC cell line or from oocytes (Musci *et al.*, 1990; Friesel and Dawid, 1991). To study the role of the FGFR in *Xenopus* embryonic development,

especially in mesoderm induction events, It was necessary to isolate FGFR cDNA clones from blastula stage, when mesoderm induction is known to occur. Therefore I constructed a cDNA library with mRNA from blastula stage (Stage 8) *Xenopus* embryos and isolated a full-length FGFR1 cDNA clone from it. The XFGFR cDNA clone isolated in our laboratory has been designated XFGFR-A3 (Chen et al., manuscript in preparation). XFGFR-A3 cDNA is a FGFR1 (flg) variant of the previously identified *Xenopus* FGFR1/flg (Musci et al., 1990) with the exception of two dipeptide deletions in the juxtamembrane region. The first is a Thr⁴²³Val⁴²⁴ (VT) deletion and the second is Pro⁴⁴¹Ser⁴⁴² (PS). The PS deletion has been previously reported in *Xenopus* (Friesel and Dawid, 1991), however, cDNA cloning and expression studies of the VT dipeptide deleted FGFR1 in *Xenopus* has not. A similar human FGFR1 variant has been isolated from live cell and the possible function of Thr⁴²⁴ has been speculated (Hou et al., 1991). Elucidation of the expression pattern of this FGFR1 variant in *Xenopus* embryos is a important step toward understanding the effects of this variant on *Xenopus* embryonic development.

We performed a quantitative analysis of XFGFR-A3 (VT deleted) and XFGFR-A2 (non-deleted) mRNA expression

during the *Xenopus* early development. Both RNase protection and RT-PCR were performed to detect XFGFR-A3 and XFGFR-A2 mRNA expression in *Xenopus* embryos. RT-PCR is an extremely sensitive method to detect specific mRNAs. This technique is very useful for rapid and simultaneous analysis of several different gene transcripts or alternative splicing products with only a few micrograms of total RNA (Hongjun *et al.*, 1990). RNase protection is a reliable and sensitive method for quantitating mRNA expression. Both XFGFR-A3 and XFGFR-A2 mRNA were shown to be expressed uniformly throughout early developmental stages (Figure 9, 10, 11). The uniform expression itself may mean both XFGFR-A3 and XFGFR-A2 have important functions during *Xenopus* embryonic development. The RNase protection experiment also showed that there was no antisense XFGFR-A3 RNA presence in the *Xenopus* embryos (Data not shown). Therefore, the control of the XFGFR-A3 function by co-expression of a complementary RNA molecule is unlikely to play an important role.

4.3. Regulation of FGFR1 Activity by PKC

Thr⁴²⁴ of XFGFR1 may serve as a phosphorylation site by a serine-threonine kinase (Hou *et al.*, 1991) and our *in vitro*

phosphorylation assay supports this hypothesis (Table 7). The results demonstrate that only the non deleted isoform was phosphorylated by PKC *in vitro* . This PKC assay is a preliminary data which was performed with purified entire fusion protein. Because the purified fusion protein sample (Figure 16) contains some contaminating bacterial proteins, we plan to repeat this experiment using a synthetic peptide to verify this result. Also, this preliminary data does not provide information about which residue is phosphorylated. We will do phosphorylated amino acid analysis on the phosphorylated fusion protein to determine which residue is phosphorylated. Additional PKC assays should be performed with the cleaved FGFR portion instead of whole fusion protein. It is important to determine the effect of phosphorylation of Thr⁴²⁴ on biological activity of the FGFR. These experiments involve expressing either XFGFR-A2 or XFGFR-A3 in a cell line that lacks endogenous FGFRs. One can activates PKC with TPA in thus transformed cell and measure its effect on FGF binding and tyrosine kinase activity. This *in vivo* study can give us more information regarding the phosphorylation of Thr⁴²⁴ residue and its influence on the biological activity of the FGFR.

Because our clone was isolated from a stage specific cDNA

library, it suggests the possibility that this potential phosphorylation site may have important consequences for signal transduction as in the case of EGF receptor (Ullrich and Schlessinger 1990). In the EGFR, it has been shown that PKC and other protein kinases can stimulate the phosphorylation of threonine⁶⁵⁴ (Thr⁶⁵⁴) located in the juxtamembrane region of EGF receptor (Lin et al., 1986; Davis and Czech, 1987). Phosphorylation of this Thr⁶⁵⁴ phosphorylation results in the lost of high affinity binding for EGF and a decrease in tyrosine kinase activity. Therefore PKC serves as a negative regulator of EGF/EGFR signalling system. Any growth factor that can stimulate PKC activity will be able to down regulate EGFR activity, a process called transmodulation (Ullrich and Schlessinger 1990).

Protein kinase C (PKC) is also activated during *Xenopus* mesoderm induction induction by FGF (Gillespie et al., 1992). However, activation of PKC in turn inhibits mesoderm induction by FGF suggesting that PKC involved in a negative feedback of FGF/FGFR signalling system as in the case of EGF/EGFR system. Therefore, Thr⁴³⁴ may be a very important residue for regulating the FGF response during *Xenopus* mesoderm induction. It means that the VT deleted FGFR1 would escape a negative feedback hence have a different impact on

mesoderm induction. Different concentrations of FGF induce different mesodermal tissues. Expressing a FGFR1 isoform that is not being turned off by PKC might be equivalent to using a higher concentration of FGF. Therefore, it is possible that the VT-deleted isoform expression is restricted to a sub-population of cells in blastula stage embryo, resulting in the generation of a different intracellular response.

4.4. Mechanism for Generating the FGFR1 Isoform

We propose that the VT dipeptide deletion was caused by the use of an alternative splice donor based on our genomic sequence analysis (Figure 12) and comparison to the cDNA and predicted amino acid sequences. This VT dipeptide is located in the juxtamembrane region and at the junction site between exon 8 and exon 9 in the human FGFR (Johnson *et al.*, 1991). Our data redefined the boundary between exon 8 and the downstream intron. According to our boundary sequence, the dipeptide deletion was VT not TV as has been previously reported (Johnson *et al.*, 1991; Hou *et al.*, 1991). Splicing is essential for the production of mature mRNAs, and according to the known intron/exon splicing mechanism and conserved splicing sequences, The VT deleted XFGFR1 form

should be the dominant product (Figure 12). But the non-deleted XFGFR1 form is in fact the major product in *Xenopus* embryos. Its mRNA accounts for more than 95 % of the whole FGFR1 mRNA population while the deleted form constitutes less than 5 %. We propose that some secondary structure was formed as guiding sequence to stabilize the splicing intermediate and results in the use of alternate splicing site which will yields the non deleted form XFGFR1.

4.5. FGFR1 Isoforms and Mesoderm Induction in *Xenopus* Embryos

The least conserved regions of RTK are the juxtamembrane region, the C-terminus, and the kinase insert sequences. It has already been shown that in other receptor tyrosine kinase the latter two regions are involved in the substrate specificity. (Cantley *et al.*, 1991). The juxtamembrane region, on the other hand, is involved in modulation of receptor function by heterologous stimuli, or receptor transmodulation. It has been shown that co-expression of a truncated FGFR1 lacking most of its cytosolic region blocked bFGF-induced signal transduction by the wild-type FGFR (Ueno *et al.*, 1992). It also has been shown that over expression of a dominant negative form of FGFR1 (cytoplasmic region

truncated) can block signal transduction by FGFR1, FGFR2 and several other family members (Amaya *et al.*, 1991). The minimum amount of truncated FGFR 1 protein necessary to block most of the wild-type receptor response is somewhere between 10 to 75 times over the amount of wild-type receptor protein. The most likely explanation is that truncated FGFRs form heterodimers with wild-type FGFRs. This heterodimer is defective in autophosphorylation (Kashles *et al.*, 1991), and hence blocks signal transduction. It is possible that in cells that express more than one variant of wild-type FGFR1, signal transduction may occur through different variant heterodimers as well as through homodimers. Evidence suggests that in cells expressing several types of FGFRs, FGFR heterodimers and homodimers may activate the cytoplasmic signalling pathway differently (Ueno *et al.*, 1992). This has led to the proposal that on the cell surface, all FGFR family members can interact with each other and that both homodimer and heterodimers are formed in order to carry out their pleiotropic functions.

At present, four members of the FGF family (aFGF, bFGF, hst/K-FGF and int-2) have been shown to have mesoderm-inducing activity (Slack *et al.*, 1987; Paterno *et al.*, 1989). It has been suggested that FGFs and/or other mesoderm

inducing signals are released from the vegetal hemisphere or from within the marginal zone, and induce the mesoderm at the equator of the embryo (Smith and Slack 1983; Slack. et al., 1984 ; Slack et al, 1987). Different types of cells have different responses to FGF. One explanation is that the availability of different members of FGFR family and their variants are regulated during the mesoderm induction process. So far, only the expression patterns of FGFR1 and FGFR2 have been reported (Yamaguchi et al., 1992, Friesel and Dawid 1991, Friesel and Brown 1992). FGFR1 mRNA is uniformly expressed at a high level throughout *Xenopus* early developmental stages. This observation is in agreement with the timing of mesoderm induction. FGFR2, on the other hand, has been found only expressed after gastrulation stages (Friesel and Brown 1992). This could mean that FGFR1 has an important function in the mesoderm induction process while FGFR2 has a role in late stages of embryonic development. However, this data can not distinguish between FGFR isoforms. Different isoforms may have unique expression patterns and specific functions.

The absence of the Val⁴²³-Thr⁴²⁴ in XFGFR-A3 may not play a direct structural role but instead may regulate the availability of a phosphorylation site. Findings from other

research groups (Tempelton and Hauschka, 1992) demonstrated that several alternatively spliced variants of FGFR1 involved in skeletal muscle cell proliferation and in the repression of terminal differentiation. Like the differential splicing in the extracellular region of FGFR1 can generate receptor variants with different ligand binding specificities (Werner 1992), the differential splicing in the juxtamembrane region could serve to diversify the intracellular responses caused by FGF/FGFR signalling system. Therefore it is possible that the intracellular signal transduction pathways activated by FGF/FGFR were changed during mesoderm induction and XFGFR-A3 may have a different function in embryogenesis. In this regard, a RNase protection experiment to determine the spatial distribution of XFGFR-A3 in *Xenopus* embryos will be very useful.

In the future, we can do *in situ* hybridization to look at localization of XFGFR-A2 and XFGFR-A3 in *Xenopus* embryos as well as use RNase protection assay. To further investigate the phosphorylation state of Thr⁴²⁴ in XFGFR-A2, we can use GST-cleaved fusion protein to do the PKC assay or use synthetic peptides to do it. We can also separately express XFGFR-A2 and XFGFR-A3 in a cell line that lacks endogenous FGFRs and analyze the cell's response to XFGFR-A2 and XFGFR-A3

expression. These experiments will give us more information about the role that XFGFR-A3 and XFGFR-A2 played in *Xenopus* development.

Chapter 5

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